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MODELLING ALPHA-SYNUCLEIN-BASED PARKINSON'S DISEASE AND STUDIES WITH CDNF

Katrina Albert

ACADEMIC DISSERTATION

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To my Dad

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ABSTRACT

The neurodegenerative disorder Parkinson's disease is often diagnosed when motor symptoms appear, at which time approximately 30% of the substantia nigra dopamine neurons have already died. The majority of disease cases are idiopathic, and there are currently no disease-modifying therapies. Since the specific molecular mechanisms underlying Parkinson's disease are still unknown, bringing treatments to the clinic has been difficult. However, in searching for both the underlying aetiology of the disease and a cure for it, animal models have brought valuable insights. Parkinson's disease animal models have been developed using toxins to cause nigrostriatal degeneration, genetic manipulation, and through the use of alpha-synuclein. Alpha-synuclein is a 14 kDa protein found abundantly in the central nervous system of vertebrates. Its importance for Parkinson's disease was clarified when it was discovered that mutations in the gene led to an autosomal dominant disease form and that it is the majority protein in what is considered a pathological marker of the disease, Lewy bodies. With these discoveries, alpha-synuclein animal models became an important tool in Parkinson's disease research.

Cerebral dopamine neurotrophic factor (CDNF) is a highly conserved protein in vertebrates with neurotrophic-like properties. It has been shown to protect dopamine neurons in rodent toxin models of Parkinson's disease and is currently in Phase I/II clinical trials for the disease. It has not been tested in alpha-synuclein animal models however, and therefore the aim of these studies was to model alpha-synuclein-based Parkinson's disease in rodents and to test whether CDNF can intervene with alpha-synuclein aggregation and has therapeutic effects in these models.

We generated two models that used alpha-synuclein specifically to attempt to model sporadic Parkinson's disease in rodents and test CDNF on: adeno-associated virus (AAV) and preformed fibrils. We used an AAV to overexpress human wild-type alpha-synuclein in rats by injecting the virus above the substantia nigra, with the end goal to test CDNF. We successfully modeled the nigrostriatal dopamine loss observed accompanied by some behavioural deficits, however, the variation in the success of the model was too high to consider it feasible to test CDNF on. This, combined with concerns about controls, led us to conclude that it may not be an ideal model of sporadic Parkinson's disease to test therapies on. Next, we used a preformed alpha-synuclein fibrils model in both mice and rats to seed endogenous alpha-synuclein after striatal injection with the goal to induce nigrostriatal dopamine neuron degeneration, progressive Lewy-like pathology spreading, and observe motor behavioural deficiencies in order to test CDNF. Here, we observed modest behavioural deficits that were ameliorated by CDNF, however the model did not result in dopamine neuron loss with the measures used. Importantly, we were able to model the spreading of Lewy body- and Lewy neurite-like inclusions that were positive for phosphorylated alpha-synuclein. After injection to the striatum there was progressive spreading of phosphorylated alpha-synuclein to the cortex and substantia nigra, which has

been shown in previous studies. While we had mildly positive effects with CDNF and from parallel *in vitro* studies we can conclude that the protein is affecting the preformed alpha-synuclein fibrils model, further studies are needed to clarify this.

We also wanted to further study CDNF, and for this we used the classic 6-hydroxydopamine (6-OHDA) toxin to selectively destroy the dopamine neurons of the nigrostriatal tract in order to model Parkinson's disease. Since CDNF has been successful in the 6-OHDA model after striatal injection, we tested injection to the substantia nigra, and also characterized the injection in naïve rats. We expected similar effects of CDNF on dopamine neurons and behaviour using nigral injection, however issues with the injection paradigm for 6-OHDA and that CDNF was given only as a single injection, resulted in only minor behavioural effects and no restoration of dopamine neurons. Although we demonstrated that when CDNF is injected to the substantia nigra of naïve rats, it is not transported to the striatum, but rather diffused around the midbrain and is taken up by nigral tyrosine hydroxylase positive neurons.

In addition to the above specific alpha-synuclein models, we used a proteasomal inhibitor, the lactacystin toxin, to characterize another model of Parkinson's disease. When lactacystin was injected above the substantia nigra of mice, we observed a buildup of alpha-synuclein, some nigrostriatal dopamine loss, neuroinflammation, and mild behavioural deficits. In general, this was repeated successfully and could be a model used for therapeutic studies.

In conclusion, we had success in modeling the progressive spreading of Lewy body-like pathology and alpha-synuclein aggregation, a phenomenon that is occurring in Parkinson's disease. However, we were not able to test CDNF in the model of AAV-alpha-synuclein overexpression due to high variation and issues with controls downregulating tyrosine hydroxylase, although we successfully recapitulated the loss and behavioural deficits observed previously. Additionally, we gained insight from CDNF studies that CDNF can prevent uptake, modulate aggregation, and ameliorate functional deficits caused by alpha-synuclein aggregation.

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
α-syn	alpha-synuclein
AADC	aromatic L-amino acid decarboxylase
AAV	adeno-associated virus
aCSF	artificial cerebral spinal fluid
ATP	adenosine triphosphate
Ca^{2+}	calcium ion
CDNF	cerebral dopamine neurotrophic factor
COMT	catechol-O-methyl transferase
CSF	cerebrospinal fluid
D1	dopamine 1 receptor
D2	dopamine 2 receptor
DAT	dopamine transporter
DIO	double-floxed inverse
DJ-1	deglycase
DNA	deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
ER	endoplasmic reticulum
GABA	gamma-aminobutyric acid
GDNF	glial line cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GFRα1	GDNF family receptor alpha 1
GPe	external globus pallidus
GPi	internal globus pallidus
GRP78	glucose-regulated protein 78
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
Iba1	ionized calcium binding adaptor molecule 1
KO	knockout
L-DOPA	L-3,4-dihydroxyphenylalanine (levodopa)
LRRK2	leucine-rich repeat kinase 2
MANF	mesencephalic astrocyte-derived neurotrophic factor
MPP+	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-4-propionoxypiperidine
MPTP	1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
NAC	non-amyloid-β component of Alzheimer's disease amyloid
NRTN	neurturin
ORF	open reading frame
PBS	phosphate buffered saline
PFF	preformed fibrils
PINK1	phosphatase and tensin homolog-induced putative kinase 1
PLA	protein ligation assay
pS129	alpha-synuclein phosphorylated at serine 129
PV	parvalbumin
Ret	receptor tyrosine kinase, encoded by Ret proto-oncogene, abbreviated from "rearranged during transfection"

SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNCA	alpha-synuclein gene
SOD1	super oxide dismutase 1
STN	subthalamic nucleus
TH	tyrosine hydroxylase
UPR	unfolded protein response
VAMP2	Vesicle-associated membrane protein 2
VMAT2	vesicular monoamine transporter 2
VTA	ventral tegmental area

ORIGINAL PUBLICATIONS

I Albert K, Voutilainen MH, Domanskyi A, Airavaara M. AAV Vector-Mediated Gene Delivery to Substantia Nigra Dopamine Neurons: Implications for Gene Therapy and Disease Models. *Genes*. 2017 Feb 8;8(2). pii: E63. doi: 10.3390/genes8020063.

II Albert K, Voutilainen MH, Domanskyi A, Piepponen TP, Ahola S, Tuominen RK, Richie C, Harvey BK, Airavaara M. Downregulation of tyrosine hydroxylase phenotype after AAV injection above substantia nigra: Caution in experimental models of Parkinson's disease. *J Neuro Res*. 2018;00:1–16. https://doi.org/10.1002/jnr.24363.

III Albert K*, Panhelainen A*, Eesmaa A, Voutilainen MH, Chmielarz P, Singh A, Luk KC, Lee VMY, Domanskyi A, Huttunen HJ, Saarma M, Airavaara M. Effects of cerebral dopamine neurotrophic factor on alpha-synuclein aggregation pathology. (manuscript)

IV Albert K, Renko JM, Mätlik K, Airavaara M, Voutilainen MH. Cerebral dopamine neurotrophic factor diffuses to subthalamic nucleus and does not undergo anterograde transport after injection to the substantia nigra. (under review *Frontiers in Neuroscience*)

V Savolainen MH, Albert K, Airavaara M, Myöhänen TT. Nigral injection of a proteasomal inhibitor, lactacystin, induces widespread glial cell activation and shows various phenotypes of Parkinson's disease in young and adult mouse. *Exp Brain Res.* 2017 Jul;235(7):2189-2202. doi: 10.1007/s00221-017-4962-z.

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1 INTRODUCTION

α-synuclein (α-syn) is a 140 amino acids long protein present throughout the brains and blood of vertebrates. Its function in the central nervous system remains somewhat elusive, however, over 20 years ago it was found that a mutation in its gene, SNCA, resulted in an autosomal dominant form of Parkinson's disease (Polymeropoulos *et al.*, 1996). This, coupled with the finding that the major component of Lewy bodies, clumps of protein found in *post mortem* brains of Parkinson's patients, is α-syn (Spillantini *et al.*, 1997) has made it a central focus in Parkinson's disease research.

Parkinson's disease is characterized by the loss of dopamine neurons, cells that produce the neurotransmitter dopamine, in the substantia nigra pars compacta and the onset of motor symptoms. Why these neurons die and result in the core motor symptoms: tremor, rigidity, bradykinesia, and postural instability, is still unknown. Though Parkinson's is a multifaceted disease with other neuronal systems being affected that result in non-motor symptoms, some which precede the motor syndrome (Hely *et al.*, 2005). Parkinson's disease is considered a neurodegenerative disease with the main risk factor being age. As there is currently only symptomatic treatment available for the disease, there has been a continual effort to develop a cure.

While the presence of abnormal accumulations of α-syn is a key aspect of Parkinson's disease, it is still unknown how, or even if, it is truly related to disease progression. This enigmatic protein has been used to model the disease in a variety of situations, from cells to animals it has been knocked out, overexpressed, or mutated – still, there are many unanswered questions. However, there are existing models that somewhat recapitulate the human sporadic form of Parkinson's disease that can, and have, been used for testing therapies.

Cerebral dopamine neurotrophic factor (CDNF) was discovered in the laboratory of Professor Mart Saarma (Lindholm *et al.*, 2007). Since then it has been tested in animal models of Parkinson's disease, amongst others, and is currently in clinical trials for the disease. CDNF is a protein with neurotrophic-like properties that may be related to an important piece of cellular machinery: the endoplasmic reticulum. CDNF's mechanism of action is still unknown however, and while it has been effective in Parkinson's animal models, its effects in animal models of α-syn-based Parkinson's disease have not been studied.

2 REVIEW OF THE LITERATURE

2.1 PARKINSON'S DISEASE

Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease, and the most common of the neurodegenerative movement disorders. Currently, over 10 million people worldwide are living with Parkinson's (Parkinson's Parkinson's Foundation, 2018). The idiopathic form of Parkinson's disease is primarily a disease of ageing, where the majority of patients are over 65 at the time of diagnosis (de Lau et al., 2004). Approximately 10-15% of cases are genetic and do not necessarily depend on age but rather specific gene mutations, whereas the other 85-90% of cases are sporadic (de Lau & Breteler, 2006). The disease is characterized by both motor and non-motor symptoms, and it is most often diagnosed when motor symptoms manifest. However, there are currently no disease-modifying therapies, only symptomatic treatment. Since it was first described in detail in the seminal essay by James Parkinson, *An Essay on the Shaking Palsy* (original publication 1817; reprinted (Parkinson, 2002)), researchers worldwide have put a large amount of effort into studying the disease pathology and attempting to stop the progression. Parkinson's disease pathology is intimately linked with one protein in particular: α-synuclein (α-syn). α-syn is known to aggregate in Parkinson's and form inclusions in the brain called Lewy bodies and neurites which may contribute to neurotransmission dysfunction and consequent cell death (Spillantini et al., 1997). These Lewy bodies are present in the brains of almost all patients *post mortem* (Gibb & Lees, 1988). Importantly, what defines all Parkinson's disease patients is the loss of dopamine neurons in the nigrostriatal system.

2.1.1 DOPAMINE NEURON DEGENERATION IN PARKINSON'S DISEASE

The nigrostriatal system consists of two primary structures: the putamen, or dorsal striatum in rodents, and the substantia nigra, which are connected in the brain by a bundle of dopamine neurons (Hattori, 1993). In Parkinson's disease, it is the dopamine neurons of the substantia nigra pars compacta that degenerate and cause the primary motor symptoms of the disease. These cardinal motor symptoms include tremor, rigidity, postural instability, as well as slowness of movement and difficulty initiating movement (Litvan, 1998). When the substantia nigra dopamine neurons die they consequently reduce dopamine terminals in the striatum (Scherman *et al.*, 1989). At the point of motor symptom manifestation however, approximately 30% of the neurons have already died in the substantia nigra and up to 60% of the dopamine terminals have been lost in the striatum (Cheng *et al.*, 2010), therefore this death is considered to occur in a "dying back" manner, where the axons degenerate first. Additionally, at this time point there is approximately 70-80% loss of dopamine content in the caudate putamen. Figure 1A shows a schematic of dopamine neuron loss in the

substantia nigra pars compacta that leads to loss of fibres connected to the caudate putamen in a healthy and Parkinson's brain. The nigrostriatal pathway is mainly responsible for voluntary movement and the suppression of involuntary movement, therefore it is no surprise that the motor symptoms of Parkinson's appear as such when these neurons degenerate. More specifically, the substantia nigra sends input to the striatum via dopamine neurons, which is part of the basal ganglia, a structure responsible for goal-directed behaviour, among others. Post-synaptic GABAergic medium spiny neurons that project directly from the substantia nigra to the striatum that mainly express dopamine 1 (D1) receptors make up the "direct pathway" and produce intrinsic excitability. Whereas GABAergic medium spiny neurons that interface indirectly to the basal ganglia and mainly express D2 receptors make up the "indirect pathway", which reduces intrinsic excitability (Surmeier *et al.*, 2014). Figure 1B shows a simplified cartoon diagram of this circuitry in the rodent brain. These pathways are thought to work in concert to both activate and suppress movement via dopaminergic input (Cui *et al.*, 2013).

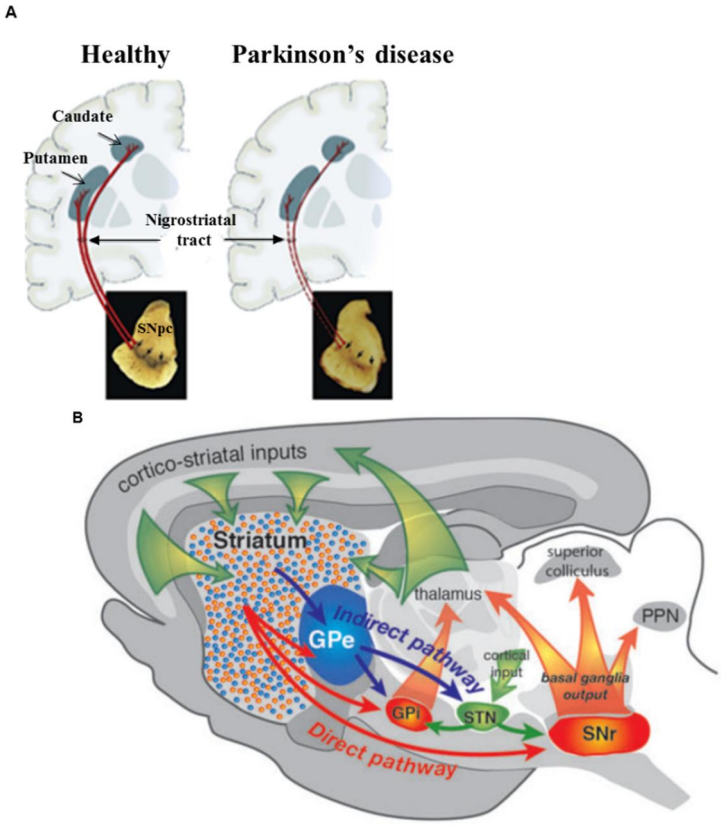


Figure 1. (A) Schematic diagram of a healthy brain where the substantia nigra pars compacta (SNpc) dopamine neurons are intact (black arrows), and the pathway to the caudate putamen, the nigrostriatal pathway, is also intact (red connecting lines); the Parkinson's disease diagram shows that the dopamine neurons of the substantia nigra pars compacta are lost and the nigrostriatal pathway degenerates (dotted red line). Modified from (Dauer & Przedborski, 2003); reprinted with permission from the copyright holder. (B) Diagram of the rodent brain connections for the direct and indirect pathways between the substantia nigra and striatum showing dopamine neuronal connections (blue), GABAergic neuronal connections (red), as well as glutamatergic (green)

neuronal connections. D1 receptor GABAergic neurons that synapse onto the internal globus pallidus (GPi) make up the direct pathway, and D2 receptor GABAergic neurons that synapse onto the external globus pallidus (GPe) make up the indirect pathway. SNr=substantia nigra reticulate, STN=subthalamic nucleus, PPN=pendunculopontine nucleus. Modified from (Gerfen & Surmeier, 2011); reprinted with permission from the copyright holder.

The progressive death of substantia nigra dopamine neurons has led researchers to ask why they are the most vulnerable, particularly when the adjacent dopamine neurons of the ventral tegmental area (VTA) show more resilience in Parkinson's disease (Alberico *et al.*, 2015), and other dopamine neurons such as those in the hypothalamus show no loss in patients (Matzuk & Saper, 1985). Although the neurotransmitter dopamine itself may be a contributing factor to this vulnerability due to the fact that it is rapidly oxidized and may lead to increased free radicals and further mitochondrial dysfunction (Berman & Hastings, 1999), it is clear that it is not the only factor since dopamine neuron death in Parkinson's disease is not happening globally but rather to specific subsets of cells. A more likely theory relates to the activity of substantia nigra pars compacta dopamine neurons. These cells have pacemaking activity, meaning they have slow intrinsic activity (Grace & Bunney, 1983; 1984b) which is most likely used to keep levels of dopamine around a certain threshold. Additionally, burst firing of these neurons occurs via excitatory stimuli and may affect dopamine release in the striatum (Grace & Bunney, 1984a). Put simply, both the pacemaking and burst activity requires calcium ion (Ca^{2+}) currents to be constantly in flux in these cells, requiring high amounts of ATP and therefore well-functioning mitochondria (Surmeier *et al.*, 2011; Surmeier & Schumacker, 2013). In Parkinson's disease, mitochondria are known to be dysfunctional (Mizuno *et al.*, 1989) and therefore any excessive bioenergetic load may be even more detrimental. Also, it is important to note that in normal ageing, there is an increase in mutations in mitochondrial DNA (Prtenjaca & Hill, 2011), and age is a top risk factor in Parkinson's. Additionally, levels of Ca^{2+} itself can induce oxidative stress (Sulzer, 2007), and it has been shown that substantia nigra dopamine neurons have a low capacity to buffer against high levels of Ca^{2+} (Foehring *et al.*, 2009). It has been shown experimentally that dopaminergic neurons of the substantia nigra pars compacta differ from dopaminergic neurons of the VTA area in that they require more energy, have more mitochondria, have higher production of reactive oxygen species (Pacelli *et al.*, 2015), and that they have a complex set of axons with a large number of terminals (Matsuda *et al.*, 2009; Pacelli *et al.*, 2015). This high energy demand due to the pacemaking activity and axonal arborization, as well as other potentials for reactive oxygen species, make these neurons more prone to stressors. This can include endoplasmic reticulum (ER) stress and dysfunction in proteasome degradation pathways due to protein buildup (Ryu *et al.*, 2002; Holtz & O'Malley, 2003) or environmental toxins (Tanner & Langston, 1990), which are linked to Parkinson's disease. Dopamine neurons of the substantia nigra pars compacta also exhibit Lewy pathology which may precede cell death, discussed in greater detail later. In general, the vulnerability of substantia nigra dopamine neurons to internal and external stress may be the reason they are preferentially degenerating in Parkinson's disease.

2.1.2 OTHER NEURONS INVOLVED IN PARKINSON'S DISEASE

Dopamine neurons are not the only neurons that degenerate in Parkinson's disease — other affected neurons include the dorsal motor nucleus of the vagus, locus coeruleus, raphe nuclei, basal forebrain, nucleus basilus of Meynert, enteric neurons, and olfactory bulb (Sulzer & Surmeier, 2013). Interestingly, the neurons of these areas also show pacemaking activity (Kang & Kitai, 1990; Goldberg *et al.*, 2012; Sanchez-Padilla *et al.*, 2014), and some exhibit complex axon branching similar to the dopamine neurons of the substantia nigra pars compacta (Surmeier *et al.*, 2017b). These areas of the central nervous system, as well as parts of the peripheral nervous system, also present Lewy pathology. This is important since it relates to the non-motor symptoms of Parkinson's disease, which are not only important clinical features but may precede motor symptoms (Schapira *et al.*, 2017). Table 1 shows affected areas and their corresponding symptom in the disease, in all cases there has been evidence of Lewy pathology (Dickson *et al.*, 2009).

Table 1. *A simplified table of areas of the nervous system that are mainly affected in Parkinson's disease and their corresponding non-motor symptoms.*

Brain area	Neurons affected	Symptom	Reference
Dorsal motor nucleus of the vagus	Sympathetic ganglia, enteric neurons*	Gastrointestinal dysfunction (ex. constipation)	(Greene, 2014)
Olfactory bulb	Mitral cells	Olfactory dysfunction (reduction or loss of smell)	(Pearce *et al.*, 1995)
Locus coeruleus	Noradrenergic neurons	Depression	(Frisina *et al.*, 2009)
Basal forebrain	Cholinergic neurons*	Cognitive defects and dementia	(Nakano & Hirano, 1984)
Brain stem, hypothalamus, cortex	Noradrenergic, serotonergic neurons*	REM sleep disorder	(Boeve *et al.*, 2007)
Cortical layers, hippocampus	Cholinergic neurons*	Dementia	(Halliday *et al.*, 2014) (Hall *et al.*, 2014)

*dopamine neurons are also affected

The non-motor symptoms in Table 1 are the often mentioned prodromal symptoms of Parkinson's disease (Schapira & Tolosa, 2010), for example, olfactory deficits are observed in 90% of Parkinson's patients prior to a definitive diagnosis (Bohnen *et al.*, 2008). Although others, such as cognitive decline, may come after the disease diagnosis (Aarsland *et al.*, 2003). Additionally, it is evident that these symptoms are generally not alleviated by current dopamine-related treatments and in fact may compromise a patient's quality of life more so than the motor symptoms (Hely *et al.*, 2005). Therefore, the non-motor symptoms of Parkinson's disease represent an important area for future disease therapy.

2.1.3 CURRENT TREATMENTS IN PARKINSON'S DISEASE

As mentioned, there is currently only symptomatic treatment for Parkinson's disease and no intervention has been able to accomplish

significant disease progression modification. Though there have been successful ways of alleviating symptoms, and in particular drugs that increase dopamine in the brain or deep brain stimulation have been extensively utilized.

L-DOPA (L-3,4-dihydroxyphenylalanine), or levodopa, is one of the most common therapies for Parkinson's patients and is recommended throughout the disease. It can be taken orally or via infusion pump to the jejunum since it crosses the blood-brain barrier and once it enters the brain it is converted to dopamine by aromatic L-amino acid decarboxylase (other names include dopa decarboxylase) (Birkmayer & Hornykiewicz, 1998). Though higher doses are associated with dyskinesia, it is still widely used to treat the motor symptoms of Parkinson's disease (Birkmayer & Hornykiewicz, 2001). Additionally, dopa decarboxylase inhibitors and catechol-O-methyltransferase (COMT) inhibitors are used as adjunct therapies with levodopa to reduce its side effects and increase its efficacy, respectively. Since orally taken levodopa metabolizes rapidly in the periphery, resulting in circulating dopamine, this causes side effects. Therefore, combination with a dopa decarboxylase inhibitor is used to counteract this as it will inhibit metabolization of levodopa into dopamine; this only occurs in the periphery and not in the brain since dopa decarboxylase inhibitors do not cross the blood-brain barrier (Thanvi & Lo, 2004). However, when dopa decarboxylase is inhibited, other enzymes may compensate, therefore COMT inhibitors are used as an additional drug (Mannisto & Kaakkola, 1990). COMT inhibitors act to increase the bioavailability of levodopa and consequently increase dopamine levels in the striatum (Mannisto *et al.*, 1988). Dopamine agonists are also used as a therapy to increase dopamine and alleviate motor symptoms either alone or in combination with levodopa (Shannon *et al.*, 1997). However, these agonists act on dopamine receptors in the periphery and may induce more severe side effects (Weintraub *et al.*, 2006), and in general levodopa is more commonly used (LeWitt, 2008).

In addition to dopamine system-related therapies it should be mentioned that there are several other classes of drugs that have been tested in Parkinson's patients. Adenosine (Bara-Jimenez *et al.*, 2003; Factor *et al.*, 2013), adrenergic (LeWitt *et al.*, 2012) and glutamate receptor antagonists (Emre *et al.*, 2010; Kobylecki *et al.*, 2014; Wang *et al.*, 2018), serotonin receptor agonists (Goetz *et al.*, 2007), Ca^{2+} channel blockers (Rees *et al.*, 2011; Parkinson Study Group, 2013), a glucagon-like peptide agonist (Athauda *et al.*, 2017), iron chelators (Devos *et al.*, 2014), and anti-inflammatory compounds (Ninds Net-Pd Investigators, 2008) have all been tested in small to large groups of patients with some success on symptoms of the disease.

Deep brain stimulation is a surgical procedure approved to treat Parkinson's disease that involves implanting a device into the internal globus pallidus, subthalamic nucleus (STN), or ventral intermediate nucleus of the thalamus to stimulate these areas electrically; the area of implantation depends on the primary symptom the physician aims to alleviate (Kringelbach *et al.*, 2007). Deep brain stimulation has the advantage of being both controlled and reversible, adding safety to efficacy. Its use results in positive effects on motor symptoms of Parkinson's disease patients,

particularly since it is mostly used in those who are resistant to dopamine replacement therapy.

2.1.4 PROGRESS IN PARKINSON'S DISEASE-MODIFYING THERAPY

In addition to these established forms of treatment, other strategies have been developed to accomplish dopamine neuron restoration that not only alleviates symptoms but also stops or reverses disease progression. One avenue has been the use of stem cells which were successful in animals (Svendsen *et al.*, 1997) and are currently in or have been in clinical trials (Barker *et al.*, 2017). More specifically, midbrain dopamine neuron progenitors are derived from embryonic stem cells or induced pluripotent stem cells and implanted into the striatum of patients (Polgar *et al.*, 2003). Although immunosuppression is needed in human patients to prevent the rejection of these cells by the immune system, and tumorigenesis is also a concern (Olanow *et al.*, 2003). Also, a well-known outcome in the field was the result that grafted dopamine neurons in the Parkinsonian patients' brains began to develop Lewy pathology (Kordower *et al.*, 2008; Li *et al.*, 2008). Therefore, while stem cell therapy is potentially promising since there are several ongoing clinical trials (NCT02538315, 2015; NCT03309514, 2017), caution needs to be taken.

Gene therapy with adeno-associated viral (AAV) vectors is another line of research that has brought potential new treatments to clinical trials for Parkinson's disease. The safety of AAVs is clear since they are non-replicating vectors that have not shown an inflammatory response (Vandendriessche *et al.*, 2007), and they can maintain long-term expression in neurons, therefore making them an optimal choice for therapy in human diseases (Lim *et al.*, 2010). Two proteins in particular have been important for Parkinson's disease treatment in order to alleviate symptoms and restore dopamine neurons: glial cell line-derived neurotrophic factor (GDNF) and its related factor neurturin (NRTN). These are neurotrophic factors, proteins that both regulate growth of developing neurons and continue to maintain adult neurons, as well as showing protection and repair of damaged neurons in animals (Airaksinen & Saarma, 2002). Administration of AAV-GDNF to the striatum was able to restore motor behaviour and dopamine neurons of the nigrostriatal system in a rat toxin model of Parkinson's disease (Wang *et al.*, 2002) and delivery to the substantia nigra of monkeys showed positive effects on the dopaminergic system (Johnston *et al.*, 2009). Currently, AAV2-GDNF is in a clinical trial for Parkinson's disease (NCT01621581, 2012); previously, infusion of the protein alone to the putamen has yielded mixed results due to the protein not being able to reach dopamine neurons or problems with delivery, but in general, GDNF seems to have a positive effect on symptoms and outcome (Nutt *et al.*, 2003; Lang *et al.*, 2006). AAV-NRTN, a neurotrophic factor in the same family as GDNF (Airaksinen & Saarma, 2002), has also been studied extensively for Parkinson's disease. AAV2-NRTN was successfully tested in rats in terms of safety and protection in a toxin model of Parkinson's disease (Bartus *et al.*, 2011a). Two trials were conducted: AAV-NRTN was delivered to the putamen alone (Marks *et al.*, 2010) or to both the putamen and substantia nigra (Olanow *et al.*, 2015).

However, neither trial showed benefits of NRTN over sham surgery – although a later analysis indicated that patients with an earlier diagnosis had a more positive response than those diagnosed later (Bartus & Johnson, 2017). Additionally, there are ongoing clinical trials for AAV-aromatic L-amino acid decarboxylase (AADC) (NCT02418598, 2015) and a previous trial for AAV-glutamic acid decarboxylase (LeWitt *et al.*, 2011) that have shown promise as a treatment for Parkinson's disease. However, the question of whether AAV injection to the striatum, substantia nigra, or both needs to be carefully considered since when the nigrostriatal system degenerates it begins with the axons of the striatum and continues to the cell bodies of the substantia nigra. Also, spreading of the protein using AAV delivery needs to be considered (Bartus *et al.*, 2011a): In the previous AAV2-NRTN trial, the coverage estimation was 20% of the putamen (Bartus *et al.*, 2011b), and in the previous AAV-AADC trial distribution of the enzyme was estimated at approximately 25% of the putamen (Mittermeyer *et al.*, 2012). In general, however, the safety and long-lasting expression of AAVs has made them promising avenues for not only symptomatic treatment, but also as a disease-modifying therapy. And while using drugs and surgical intervention has been of great benefit for Parkinson's disease treatment, researchers, physicians, and patients continue to search for a cure.

2.1.5 GENETIC FORMS OF PARKINSON'S DISEASE

Though the majority of Parkinson's cases are sporadic, a small portion have a genetic cause. These are monogenic forms that arise from the mutation, either dominant or recessive, of a single gene. There are currently 17 identified PARK chromosomal loci mutations which have been confirmed to result in a Parkinsonian phenotype, (Klein & Westenberger, 2012), and in total 26 loci have been identified as a risk of developing Parkinson's disease (Lill, 2016). For the purpose of this thesis only the 6 that have the clearest evidence as being linked to monogenic and heritable Parkinson's disease will be discussed.

LRRK2 (leucine-rich repeat kinase 2; PARK8) mutations are the most common autosomal-dominant, as well as the genetic cause of sporadic, form of Parkinson's disease (Lesage *et al.*, 2006; Ozelius *et al.*, 2006). This form is mid-to-late onset and does not always present with Lewy pathology (Giasson *et al.*, 2006). Parkin (PARK2) causes an autosomal-recessive form of the disease and often begins when the patient is 30-40 years old; homozygous carriers can show onset before 21 years of age (Kitada *et al.*, 1998). Parkin patients are mostly lacking Lewy bodies. PINK1 (phosphatase and tensin homolog (PTEN)-induced putative kinase 1; PARK6) is the second most frequent autosomal-recessive form with a phenotype similar to Parkin. Most PINK1 mutations are loss-of-function and may work in concert with Parkin to induce mitophagy (Valente *et al.*, 2004). DJ-1 (deglycase, PARK7) is also autosomal recessive and presents similarly to both Parkin and PINK1 with early onset Parkinson's disease, though this is the rarest of the three (Bonifati *et al.*, 2003). Less is known about DJ-1 but it has been shown that the mutated form does not fold properly and is degraded quickly (Moore *et al.*, 2003). Autosomal recessive mutations in ATP13A2 (PARK9) have been

demonstrated to result in juvenile onset Parkinson's that progresses quickly, it also presents with other symptoms and is known as Kufor-Rakeb syndrome (Ramirez *et al.*, 2006).

Last, but not least, is the SNCA (PARK1/4) mutation. Mutations in SNCA cause autosomal dominant Parkinson's disease that has an onset of less than 50 years of age (Polymeropoulos *et al.*, 1996). This form of the disease progresses quickly and shows Lewy pathology that spreads throughout similarly affected brain areas in sporadic Parkinson's such as the substantia nigra and locus coeruleus (Polymeropoulos *et al.*, 1996). SNCA is in fact the gene coding the α-syn protein and duplications (Chartier-Harlin *et al.*, 2004) and triplications (Singleton *et al.*, 2003) of SNCA result in Parkinson's of increasing severity (Fuchs *et al.*, 2007). In general, though the genetic forms of Parkinson's disease are far less common, discovering these genes and their effects have given valuable information about disease aetiology.

2.2 ALPHA-SYNUCLEIN

As mentioned, genetic mutations of the SNCA gene result in an autosomal dominant form of Parkinson's disease, and α-syn is the main component of Lewy bodies (Spillantini *et al.*, 1997), which are found in almost every idiopathic case of the disease (Gibb & Lees, 1989). α-syn is present in healthy humans but has become an extensively studied protein in relation to Parkinson's disease for the above reasons. Since α-syn is considered the main pathogenic protein of Parkinson's disease a majority of research focus has been placed on it.

2.2.1 ALPHA-SYNUCLEIN FUNCTION AND KNOCKOUT MODELS

Synuclein was first isolated from the cholinergic vesicles at the synapse and nuclear envelope of *Torpedo* (Maroteaux *et al.*, 1988), thus the name "synuclein". It was also found in the zebra finch where it functions in song learning (George *et al.*, 1995), pointing to a potential role in synaptic plasticity. Both of these proteins were found to be the homologue of human α-syn and along with β- and γ-synuclein it makes up the synuclein family (Jakes *et al.*, 1994). α-syn is a 140 amino acid protein that is present throughout the central and peripheral nervous systems, though it is also present in red blood cells (Barbour *et al.*, 2008). α-syn is an abundant protein, being 1% of all cytosolic protein in the brain (Stefanis, 2012). More specifically, it is localized at the nerve terminal: a study using transgenic mice expressing human wild-type α-syn fused to green fluorescent protein (GFP) were imaged *in vivo* with multiphoton microscopy. The authors were able to visualize neurons in the live mouse brain and determine that the majority of α-syn was located at presynaptic terminals (Unni *et al.*, 2010). This has already been shown similarly in humans; healthy human brain sections were immunostained for α-syn and synaptophysin, a synaptic vesicle protein, and it was observed that they colocalized in neurons (Kahle *et al.*, 2000).

α-syn's localization at the synapse has been known for over 20 years, however its normal function has not been completely elucidated – though it seems to be related to the SNARE complex, which plays a crucial role in

neurotransmitter release (Sudhof, 2013). It has several known interacting proteins at the presynaptic terminal: synaptobrevin-2 (Burre *et al.*, 2010), synapsin III (Zaltieri *et al.*, 2015), synphilin (Engelender *et al.*, 1999; McLean *et al.*, 2001; Ribeiro *et al.*, 2002), vesicular monoamine transporter 2 (VMAT2) (Guo *et al.*, 2008), dopamine transporter (DAT) (Butler *et al.*, 2015), and serotonin transporter (Wersinger *et al.*, 2006). It also regulates tyrosine hydroxylase (TH) (Perez *et al.*, 2002), the rate-limiting enzyme of dopamine and other catecholamine synthesis, and the membrane interaction of Rab3 (Chen *et al.*, 2013). Additionally, it binds to DJ-1 (Zondler *et al.*, 2014), as well as tubulin to regulate microtubules (Lee *et al.*, 2006). Lastly, it has been found to enhance phosphorylation of tau, a protein also involved in Alzheimer's disease (Jensen *et al.*, 1999; Haggerty *et al.*, 2011). There are also suggested roles of α-syn in lipid transport (Fortin *et al.*, 2004). However, since there is no α-syn in non-vertebrates (ex. *Saccharomyces*, *Drosophila*, or *C. elegans*) (George, 2002), this has made it difficult to ascertain the precise function and true importance of this protein.

One of the most common and useful tools for determining a protein's function *in vivo* is by studying knockout (KO) animals, where the protein is either removed completely or in a specific tissue from an animal. Since α-syn has a plethora of binding proteins and seems to be important in synaptic transmission, naturally a mouse KO was expected to have clear deficits in, for example, neurotransmitter release and/or synaptic plasticity. There have been single α-syn KO, double KO of α+β-syn, and triple KO of α+β+γ-syn in mice. However, the deficits have not been so pronounced. The single KO mice have reduced dopamine content in the striatum and amphetamine-induced locomotor activity, though interestingly, they displayed a faster recovery of dopamine release. Although, they are viable and do not show loss of nigrostriatal dopamine neurons (Abeliovich *et al.*, 2000). By contrast, a different group showed that deletion of the SNCA gene resulted in fewer TH+ neurons in the substantia nigra during mouse development (Garcia-Reitboeck *et al.*, 2013). In another single KO study, the authors also found that the mice had a lower capacity of dopamine storage, but that there was an increased rate of refilling of the readily releasable pool of vesicles, possibly acting as a compensatory mechanism (Yavich *et al.*, 2004). The double and triple KO mice were bred to account for possible compensation in the nervous system by β- and γ-syn. In the double KO for α+β-syn, mice were also viable and fertile (Chandra *et al.*, 2004). As with the single α-syn KO, there were no gross changes in the brain and importantly the number of TH+ cells in the substantia nigra was similar to wild-type littermates. Although striatal levels of dopamine were decreased as well. In general, there were no significant changes of the synapses or vesicles. A notable change was the increased protein levels of γ-syn, complexin, and 14-3-3ε, and decrease in of 14-3-3ζ. γ-syn's increase, although likely a compensatory mechanism, was considered not overly interesting since the levels in the adult brain are already low (Clayton & George, 1998). However, complexin is also a small, presynaptic protein involved in neurotransmitter release (Reim *et al.*, 2001) and the 14-3-3 proteins are similar in homology to α-syn (Ostrerova *et al.*, 1999). The triple synuclein KO mice were similar to wild-type mice in survival and overall brain architecture (Anwar *et al.*, 2011). There were also no changes in number of TH+ cells in the substantia nigra pars compacta or VTA, as well as TH

fibres in the striatum. Synaptic proteins, the SNARE complex, and synapse morphology were also unchanged. As with the other KO mice, the levels of striatal dopamine were significantly decreased compared to the wild-type. For behaviour, the triple KO mice performed worse on an accelerated rotarod and had increased exploratory activity in a "novel, non-anxiogenic environment", the latter of which the authors hypothesize is related to hyperdopaminergic activity. This could be due to the fact that even though dopamine content was decreased in the striatum, when dopamine release was stimulated in striatal slices, the KO had a higher extracellular amount than wild-type mice, which was not due to faster reuptake. Another behavioural experiment performed sheds light on this: when amphetamine was given to the KO mice, a drug that increases extracellular dopamine by reverse transport at the vesicles (Sulzer *et al.*, 1995), they had reduced stimulated motor activity compared to wild-type. This could indicate that there is less dopamine in presynaptic vesicles in the triple synuclein KO. In general, since it is not clear that there would be a major deficit on the dopamine system of KO mice, it seems that α-syn, and the rest of the synuclein family, is having a minor effect on dopamine release resulting in a decrease in overall striatal dopamine. Lastly, a study from Burre and colleagues (Burre *et al.*, 2010) revealed that α-syn binds to synaptobrevin-2/VAMP2 to enhance SNARE complex assembly. Further, the authors used the triple synuclein KO to study *in vivo* effects and found an age-dependent neurological deficit, decreased survival, and a significant decrease in synaptobrevin-2 – in other words, only the aged mice (~1 year) exhibited these characteristics, implicating synucleins in neuronal function over time. From these interaction and KO studies, it is clear that α-syn is functioning at the presynaptic cleft and this may be where its involvement with dopamine neurotransmission is the most important.

2.2.2 ALPHA-SYNUCLEIN AND DOPAMINE

As discussed above, α-syn interacts with various proteins related to dopamine and a marked decrease of striatal dopamine content is observed in the KO mice. Importantly, through its direct interaction with TH, it was shown that in a dopaminergic cell line (MN9D) where α-syn was overexpressed, there was a decrease in TH activity which resulted in reduced dopamine synthesis; this effect occurred without a decrease in overall TH protein level (Perez *et al.*, 2002). The mechanism of TH activity reduction was via decrease in phosphorylation of Ser40 after overexpression of α-syn in MN9D cells, where phosphorylation of Ser40 on TH is considered to significantly contribute to dopamine synthesis (Peng *et al.*, 2005). To complement the *in vitro* studies, studies have found that there is an age-dependent relationship between α-syn and TH in healthy humans and monkeys, where TH decreases over time whereas α-syn increases over time in the soma of nigral neurons (Chu & Kordower, 2007).

α-syn's interaction with VMAT2 has also been explored further using overexpression vectors in mice. α-syn was overexpressed in the substantia nigra neurons of mice that were deficient in VMAT2, and this resulted in increased dopamine degeneration compared to wild-type, which was reversed by silencing of TH (Ulusoy *et al.*, 2012). The hypothesized mechanism of this

finding is that since VMAT2's normal function is to sequester dopamine to reduce its oxidation, the reduction in VMAT2 led to an increase in dopamine in the substantia nigra neurons, thus causing cell death due to higher amounts of dopamine being present. Since this effect was reversed by loss of TH, thereby bringing the amount of dopamine closer to normal levels, it stands to reason that dopamine itself was causing more severe neurodegeneration in the mice. However, this theory is difficult to rectify with current knowledge since levodopa has not shown clear indication of increased disease progression in Parkinsonian patients (Olanow, 2015).

In addition to VMAT2, DAT as an interaction partner of α-syn has been studied but the results have been conflicting for the exact outcome (Butler *et al.*, 2017). DAT has been shown to interact with α-syn, including in *in vivo* studies using a proximity ligation assay (PLA) that detects protein-protein interactions (Bellucci *et al.*, 2011a). DAT is an important protein that regulates dopamine levels in the brain by removing it from the extracellular space and replenishing it in the neuron, thus DAT is able to both terminate dopamine neurotransmission and maintain it at the synapse. An *in vitro* study has shown that increasing levels of α-syn results in decreased uptake of dopamine via DAT (Swant *et al.*, 2011). This was also demonstrated *in vivo* where authors injected *E. coli*-produced recombinant human α-syn directly into the striatum of α-syn KO mice and observed a decrease in dopamine uptake as well as evoked overflow six days after injection, likely related to DAT (Pelkonen *et al.*, 2013). Related to this, in a mouse model overexpressing α-syn, there was a significant increase in striatal dopamine in the mutant mice compared to the wild-type mice at six months of age, which resulted in decreased dopamine tissue content and TH expression in the striatum at fourteen months old. Although this was not necessarily related to DAT since reuptake was unchanged between the genotypes (Lam *et al.*, 2011). Additionally, there has been a conflicting report of the α-syn-DAT outcome: that DAT actually increases dopamine uptake when human α-syn and human DAT are co-expressed in cells (Lee *et al.*, 2001). In general, α-syn's links to dopamine at the presynaptic cleft continues to be a focus for researchers, however it is still not known exactly how these interactions may be relevant to dopamine neuron death or modulation of neurotransmission in Parkinson's disease.

2.2.3 ALPHA-SYNUCLEIN STRUCTURE

The structure of α-syn has received attention for a number of reasons. Different parts of the α-syn protein contribute to its various aggregation, functional, and binding properties, also post-translational modifications and mutations of the protein are important, particularly in disease. Additionally, there has been debate in the field about the physiological structure of α-syn and how it may contribute to aggregation and neuronal dysfunction. A widely cited view is that α-syn exists in an equilibrium between two states in the brain: it is natively unfolded in the cytosol (Burre *et al.*, 2013), but in a helical, multimeric state when bound to membranes (Burre *et al.*, 2014). This theory posits that α-syn mainly exists as a monomeric 14 kDa protein that is intrinsically disordered and prone to aggregation. However, this theory has

been criticized due to the fact that these experiments were performed using recombinant α-syn protein purified using *E. coli* systems with sample heating and/or denaturing, which may not represent relevant physiological conditions; and it is well-known that proteins produced in *E. coli* are often not folded as they would normally be *in vivo*. Therefore, when researchers used samples of brain tissue, human cells, and cell lines without denaturation they found that α-syn principally occurred as a folded tetramer of approximately 58 kDa in size (Bartels *et al.*, 2011). To add to this, it was found that tetrameric α-syn has vesicle trafficking functions at the synapse under normal conditions (Wang *et al.*, 2014). Therefore, in opposition to the above theory, researchers hypothesize that α-syn rather exists more often as an α-helical folded tetramer in equilibrium with the unfolded monomer, and that the tetramer is resistant to aggregation. To avoid confusion in this thesis, I have defined the terms in Box 1 which shows a list of glossary terms used here when referring to α-syn structure throughout.

Box 1. *α-synuclein structure terms.*

monomer	14 kDa protein, unfolded, prone to aggregate
tetramer	58-60 kDa protein, α-helical folded, resists aggregation
oligomer	60+ kDa protein, may cause cell dysfunction if misfolded
fibril	fibrillar, β-sheet structure, considered aggregate and may cause cell dysfunction
Lewy body or neurite	cluster of proteins, of which α-synuclein is the main component

The N-terminal region of monomeric α-syn contains an amphipathic α-helix formed by an 11-residue repeat sequence related to phospholipid membrane binding (Bussell & Eliezer, 2003), and it is here that the missense mutations are occurring. The acidic C-terminus consists of mostly glutamate and is generally where most of the protein interactions take place, as well as protection from aggregation (Murray *et al.*, 2003) (Figure 2). In between the N- and C-termini is the non-amyloid-β component of Alzheimer's disease amyloid (NAC) region, so named since it was first purified in amyloid plaques of Alzheimer's patients. It consists of a 35 amino acid sequence that is hydrophobic, and the human form is prone to aggregation, but interestingly not the mouse (Ueda *et al.*, 1993). Figure 2 represents the schematic sequence of α-syn. In addition to its normal structure under physiological conditions, during pathological conditions it can adopt a β-sheet formation that is related to aggregation, fibril formation, as well as Lewy bodies (Rochet *et al.*, 2000; Ding *et al.*, 2002).

These pathological conditions can come from overexpression or high amounts of α-syn or from mutations of the protein. The following missense mutations have been found in α-syn to cause Parkinson's disease: A30P (Kruger *et al.*, 1998), E46K (Zarranz *et al.*, 2004), H50Q (Proukakis *et al.*, 2013), G51D (Kiely *et al.*, 2013), A53E (Pasanen *et al.*, 2014), and A53T (Polymeropoulos *et al.*, 1997). In addition to confirming α-syn's importance in Parkinson's disease, their use in modeling the disease has become an important tool.

Another structural aspect of α-syn is its forming of oligomers or fibrils. The formation of α-syn aggregates is considered to occur by natively unfolded α-syn monomers forming intermediate aggregated oligomers, and then these

form into insoluble fibrils, the latter being toxic as it builds up (Fink, 2006). Fibrillization of α-syn is considered the main aggregation form in Parkinson's disease. However, the formation of oligomer intermediates is also considered an important step in the process, and this form may be equally or even more detrimental than the fibrillar form when the oligomer is misfolded. Oligomers in this case being approximately 80 kDa in size, in other words larger than the approximate 60 kDa tetramer (Dettmer *et al.*, 2013). Studies have shown that these oligomers can cause cell death *in vitro* after transfection of the cells and in non-mammalian models using expression vectors of α-syn (Danzer *et al.*, 2007; Karpinar *et al.*, 2009). In rodents, loss of TH in the substantia nigra neurons was demonstrated when mutant α-syn that favours oligomer formation was injected into the substantia nigra via lentiviral vector (Winner *et al.*, 2011), and in a mouse model that received α-syn oligomers directly to the brain showed motor and non-motor deficits, as well as olfactory and dopamine degeneration (Fortuna *et al.*, 2017). As well, a study in transgenic mice with a mutation that preferentially forms oligomers had defects in synaptic function, loss of dendritic spines, and some behavioural deficits (Rockenstein *et al.*, 2014). However, others have shown that fibrils are in fact the most harmful strain of α-syn (Peelaerts *et al.*, 2015). In relation to this, the tetrameric form must also be considered. When the levels of monomers that go on to form insoluble aggregates increases, the levels of tetramers decrease: this can be achieved by mutations that decrease the tetramer:monomer ratio and may lead to disease initiation (Dettmer *et al.*, 2015). In fact, this was demonstrated in a mouse model where mutations that shifted tetrameric α-syn to monomeric α-syn resulted in insoluble inclusions, a motor syndrome where mice showed abnormal gait and tremor, and some degeneration in the nigrostriatal dopamine system (Nuber *et al.*, 2018). Although interestingly, in this model the pathology was not only in the striatum and midbrain, but also observed in the cortex, indicating that cortical layers and/or their connections may also be involved in motor symptoms. Additionally, α-syn can undergo several post-translational modifications. These are summarized in Table 2.

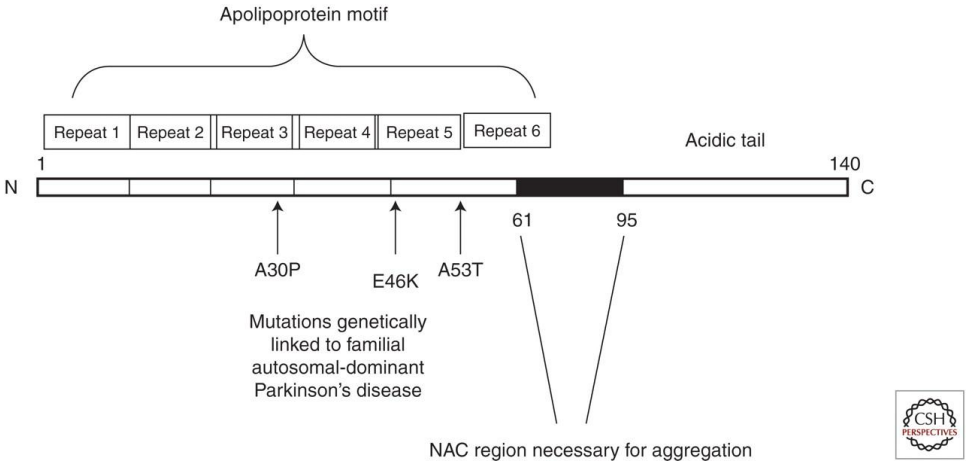


Figure 2. A simplified schematic sequence of α-synuclein showing the N- and C-termini as well as the NAC region. Common mutations are also indicated. Modified from (Stefanis, 2012); reprinted with permission from the copyright holder, Cold Spring Harbor Laboratory Press.

Table 2. *A summary of α-synuclein posttranslational modifications and their proposed function or outcome.*

Modification	Location	Function	Reference
Phosphorylation	S87, S129, Y125, Y133, Y135	Inhibition of aggregation	(Waxman & Giasson, 2008) (Negro *et al.*, 2002)
Acetylation	N-terminal	Increases membrane affinity and helical folding	(Maltsev *et al.*, 2012)
Ubiquitination	Lysine residues (ex. K21, K23, K32, K34 may be important)	Ubiquitination in pathological conditions (ie. Lewy bodies, neurites)	(Nonaka *et al.*, 2005)
Glycation	Arginine residues (unpublished observation)	Glycation end products have been observed in Parkinson's disease brains	(Munch *et al.*, 2000)
Glycosylation	Residues 53, 64, 72, 87	Unknown; may inhibit aggregation	(Marotta *et al.*, 2012)
Nitration	Tyrosine residues	Nitrated α-syn in pathological fibrils; also shown to inhibit fibril formation	(Giasson *et al.*, 2000) (Yamin *et al.*, 2003)
Proteolysis	C-terminal truncation	Increase aggregation and fibril assembly	(Serpell *et al.*, 2000)

Phosphorylation of α-syn has been a strong research focus, particularly phosphorylation at serine 129 (pS129). This is due to the fact that there is indication that pS129 is present in Lewy bodies (Anderson *et al.*, 2006) and studies from primates have shown that it is increased during ageing in the substantia nigra (McCormack *et al.*, 2012). Interestingly, though pS129 α-syn is present in healthy human brains, particularly in the substantia nigra and nucleus basalis of Meynert (Muntane *et al.*, 2012), it has been shown to make up approximately 90% of the α-syn in brains of patients with a synucleinopathy (Fujiwara *et al.*, 2002). pS129 α-syn has been shown to have other functions *in vitro*, however it is still not fully understood whether this phosphorylation is a toxic event in itself that may increase inclusion formation or whether it precedes α-syn pathology in the brain (Oueslati *et al.*, 2010; Oueslati, 2016).

The structure of α-syn plays a critical role in both its normal and potential pathological functions, making it a continued area of research focus. Additionally, pS129 α-syn appears to be important not only in Parkinson's disease, but also other diseases that are linked to α-syn.

2.2.4 ALPHA-SYNUCLEIN IN NEURODEGENERATIVE DISEASES

While α-syn is normally present in the healthy human brain, its accumulation in to Lewy bodies and neurites in Parkinson's as well as mutations in the SNCA gene are clear indications it is related to the disease. Additionally, α-syn and Lewy bodies are present in other neurodegenerative disorders other than Parkinson's.

Lewy bodies are mostly α-syn immunoreactive (Spillantini *et al.*, 1997), but there is also ubiquitin and other proteins present in these inclusions (Goldman *et al.*, 1983; Kuzuhara *et al.*, 1988; Kawamoto *et al.*, 2002). They are considered to be the main neuropathological indicator of Parkinson's disease since they are found in most patients (Gibb & Lees, 1989), but not all (Berg *et al.*, 2014). Lewy bodies form in the cytoplasm and neurites in the neurons of the brain, and they have been found throughout the Parkinsonian brain, including the substantia nigra (Crowther *et al.*, 2000). Lewy pathology is present throughout the brains of Parkinson's patients and appears to spread from one area to another. The most famous characterization of this comes from Heiko Braak and the staging hypothesis that he and others postulated (Braak *et al.*, 2003). Braak theorized that over the time course of the disease Lewy pathology is present in an increasing number of brain areas, and interestingly that it may initiate in the autonomic nervous system, more specifically the gut and olfactory system (Hawkes *et al.*, 2007). Importantly, the brain areas that show pathology are anatomically connected, so it seems that α-syn pathology is spreading along neuronal tracts. Further evidence for spreading of α-syn came from the finding that when fetal dopamine neurons were transplanted to Parkinson's patients' brains, these healthy neurons also developed Lewy pathology (Kordower *et al.*, 2008; Li *et al.*, 2008). This led to the hypothesis of cell-to-cell transfer of α-syn and further studies *in vitro* and *in vivo* to demonstrate this phenomenon and discover possible mechanisms. Purified fibrils of α-syn have been used as a tool to elucidate the propagation of the protein and as such have been quite powerful, as will be discussed below in relation to models of Parkinson's disease. However, though there is ample evidence from models of α-syn spreading, also referred to as the prion hypothesis, how it is contributing to disease pathology is still unclear. Opponents of the staging/prion hypothesis point out that only approximately half of patients have this staging scheme (Kalaitzakis *et al.*, 2008) and that it does not correlate with disease severity (Jellinger, 2009). Additionally, the areas with the most severe Lewy pathology do not necessarily transmit large amounts of α-syn to their connected areas, for example the locus coeruleus (Burke *et al.*, 2008; Surmeier *et al.*, 2017a). As discussed above, it appears that the pacemaking and related high energy load of substantia nigra dopamine neurons makes them vulnerable to stressors in Parkinson's disease, and that is what is causing disease pathology. However, though the presence of α-syn cannot be denied but may instead be a consequence of impaired neuronal function, rather than the initial trigger of the disease. Evidence of this comes from observations that cell dysfunction may come before Lewy pathology is detected, which has been demonstrated with substantia nigra dopamine neurons (Milber *et al.*, 2012). And as such, impaired autophagy and lysosomal pathways (Pan *et al.*, 2008; Dehay *et al.*, 2013), ER stress (Lindholm *et al.*, 2006), proteasomal degradation pathways (Olanow & McNaught, 2006), have all been implicated in Parkinson's disease. A diagram indicating possible contributions to Parkinson's disease is shown in Figure 3.

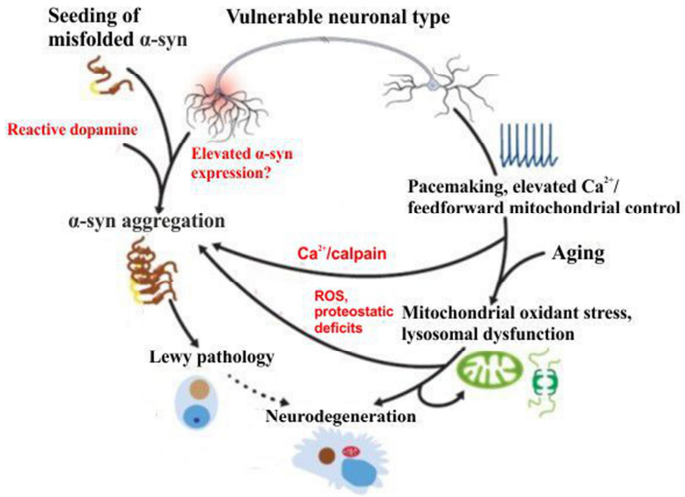


Figure 3. Potential contributions to Parkinson's disease pathophysiology. Modified from (Surmeier *et al.*, 2017a); reprinted with permission from the copyright holder.

The presence of α-syn has also been important in other neurodegenerative diseases. As mentioned, it has been found in Alzheimer's disease (Lippa *et al.*, 1998), and it has been documented in the rare parkinsonism-dementia complex of Guam (Yamazaki *et al.*, 2000). Though the most important diseases with Lewy pathology are dementia with Lewy bodies, Parkinson's disease with dementia, and multiple system atrophy. Dementia with Lewy bodies is exactly as it sounds: patients have late-onset dementia and a high degree of Lewy pathology in the cortical areas (Kosaka, 1978). Interestingly, these are similar to the α-syn-reactive Lewy bodies of the substantia nigra in Parkinson's disease patients (Spillantini *et al.*, 1998b). Parkinson's disease with dementia is very similar to Parkinson's disease, however it includes memory loss accompanied by tau inclusions, which are far less common in Parkinson's disease (Burn *et al.*, 2006). This disorder also has Lewy pathology similar to Parkinson's (Lippa *et al.*, 2007). Multiple system atrophy is clinically similar to Parkinson's disease since the core symptoms are similar to the motor symptoms observed in it (Lantos, 2001). Additionally, autonomic symptoms such as incontinence are observed, and neuropathology is also present in the substantia nigra, striatum, and locus coeruleus, as well as pontine nuclei, cerebellum, and parts of the spinal cord (Wakabayashi *et al.*, 1998). A distinctive facet of multiple system of atrophy is that the α-syn-reactive inclusions are mostly in the glial cells, particularly the oligodendrocytes (Spillantini *et al.*, 1998a). Together, these diseases are often referred to as synucleinopathies. These studies have pointed to α-syn as an important aspect of disease, and how it may relate to progression and severity continues to be a focus for research.

2.2.5 ALPHA-SYNUCLEIN AND PROTEIN DEGRADATION

Since α-syn is accumulating in disease, but not in the healthy human brain, this may be occurring due to dysfunction in systems related to protein

clearance. There are two important systems related to the degradation of proteins: the ubiquitin-proteasome system and the autophagy-lysosome pathway, and as mentioned these may be dysfunctional in Parkinson's disease as well as other neurodegenerative diseases that have an abnormal buildup of proteins (Pan *et al.*, 2008).

The ubiquitin-proteasome system is responsible for degrading most short-lived and misfolded proteins (Martinez-Vicente & Vila, 2013). Briefly, this system functions by tagging proteins with ubiquitin and then these tagged proteins are specifically degraded by a proteasome; this system functions in a highly regulated manner (Finley, 2009). The connection between the ubiquitin-proteasome system and Parkinson's disease comes from both cellular and *in vivo* models that use a proteasome inhibitor to induce death of substantia nigra dopamine neurons and buildup of α-syn (McNaught *et al.*, 2002a; McNaught *et al.*, 2002b). Importantly, mutations in parkin, which is known to cause a genetic form of Parkinson's disease, may be related to dysfunction in degradation of proteins by the proteasome resulting in impaired α-syn clearance. Parkin is a part of the machinery, the E3 ubiquitin ligase, that adds the ubiquitin chain to proteins to be sent for degradation by the proteasome (Kitada *et al.*, 1998), and it has been shown that mutations led to accumulation of α-syn in the human brain (Shimura *et al.*, 2001). Additionally, α-syn has been shown to interact with one of the most-studied proteasomes, 26S, and impair its function (Snyder *et al.*, 2003).

There are various ways in which the lysosome-autophagy pathway can clear α-syn. Generally, this occurs by ubiquitination of aggregates that membranes then form around, then they are fused with a lysosome for degradation (Yamamoto & Simonsen, 2011). Autophagy, more specifically macroautophagy, is important since it has been shown to clear aggregated α-syn (Ravikumar *et al.*, 2008), unlike the ubiquitin-proteasome system which clears soluble protein. *In vitro*, blocking autophagy results in accumulation of α-syn (Lee *et al.*, 2004), and α-syn itself has been shown to interfere with macroautophagy, causing undegraded proteins to buildup (Winslow *et al.*, 2010).

These two systems work to clear out proteins from the brain of mammals and are likely both acting on α-syn and are affected by α-syn, as well as other aggregating proteins in neurodegenerative diseases. Research continues to fully understand their role in Parkinson's disease and other synucleinopathies, and in general their contribution to disease.

2.2.6 ALPHA-SYNUCLEIN AS A BIOMARKER AND DRUG TARGET

Not only is it difficult to diagnose Parkinson's disease before the motor symptoms appear, misdiagnosis between other synucleinopathies mentioned above is also an issue. Since these disorders all present with α-syn-reactive Lewy bodies, this makes α-syn an attractive candidate for a biomarker. However, it should be noted that Lewy pathology has also been observed in asymptomatic human brains *post mortem* (Klos *et al.*, 2006).

In diagnosing Parkinson's disease several avenues have been tried to correlate disease with α-syn presence. Biopsies of the salivary gland have shown promise: one study showed that staining the submandibular gland for

α-syn resulted in Lewy body detection in 100% of Parkinson's patients, and none of the controls (Del Tredici *et al.*, 2010). Additionally, in colon biopsies of Parkinson's disease patients, 72% were positive for Lewy bodies, and no controls (Lebouvier *et al.*, 2010). Though these biopsy studies are useful, this is a much more invasive procedure and therefore looking at bodily fluids has become important in the field. Cerebrospinal fluid (CSF) samples have been used with mostly positive results, however this is also invasive. Though it has shown high specificity and sensitivity for Parkinson's disease patients (Hong *et al.*, 2010), and interestingly oligomeric (Tokuda *et al.*, 2010) and pS129 (Wang *et al.*, 2012) α-syn were increased in samples from patients. Detection in blood plasma has also been explored, however this has produced confounding results due to α-syn's presence in red blood cells (Barbour *et al.*, 2008) as well as plasma (El-Agnaf *et al.*, 2003). Though one study found that quantified α-syn not only showed similar specificity and sensitivity to CSF samples, but was also correlated with disease severity (Shi *et al.*, 2014), and a small study showed that pS129 α-syn was higher in Parkinson's disease patients than in controls (Foulds *et al.*, 2011). Currently, using blood and salivary samples are being explored, but larger studies are still needed.

Since α-syn has been shown to be differentially present in Parkinson's patients compared to healthy controls, naturally therapy targeting α-syn has become important. A study screening different compounds for their ability to reduce SNCA gene expression found that a β2-adrenergic receptor agonist was able to reduce expression without causing cell death, and interestingly, that β2 agonists were associated with a decreased risk of Parkinson's disease and antagonists with an increased risk (Mittal *et al.*, 2017). This is an interesting prospect since these drugs already have a wealth of research, are approved for use in other conditions, and readily cross the blood-brain barrier. Another route is to target the α-syn protein itself: an immunotherapy vaccine is being developed (Affiris, 2018), as well as antibodies against α-syn (Schenk *et al.*, 2017), both of these approaches have reached the clinical trial stage and have been reported to be safe, but their efficacy in reducing α-syn levels and their effect on disease outcome remains to be seen. However, outcomes of a recent trial in Alzheimer's disease showed no effects on cognitive outcomes in one study with a monoclonal antibody against amyloid-β (Salloway *et al.*, 2014) and in another there were no significant differences between placebo and the antibody treatment on free amyloid-β load (Siemers *et al.*, 2016). And in fact – the latter drug increased the amyloid-β species, $A\beta_{1\text{-}42}$, that has been found to be neurotoxic (Gold, 2017). Additionally, techniques to enhance autophagy pathways to increase α-syn clearance are being explored (Brundin *et al.*, 2017).

Clearly, researchers and clinicians must continue to search for more accurate diagnostic measures to diagnose Parkinson's disease earlier, and therefore have a higher chance of a successful disease-modifying therapy while more of the dopamine neurons are still alive. Additionally, therapies targeting α-syn as the main neuropathological protein in the disease are potentially promising, however, it is still not fully understood how α-syn affects disease progression and outcome, making animal models necessary to not only test new therapeutics, but also model Parkinson's disease aetiology and development.

2.3 ANIMAL MODELS OF PARKINSON'S DISEASE

Generally, animals do not develop Parkinson's disease as far as we understand it, though there is a syndrome observed in canines that is similar to dopamine deficiency and Parkinson's in humans (Bruyette *et al.*, 1995). However, the ability to induce Parkinson's disease-like symptoms and pathology in animals is still currently a necessity in research. There are a variety of *in vivo* models for Parkinson's disease, in organisms ranging from yeast to non-human primates. Over the last several decades, these animal models have developed from more basic toxin models where the main feature is destruction of the dopaminergic system, to more complex models that incorporate different facets of the disease such as genetic and α-syn components (Koprich *et al.*, 2017). Though non-vertebrate Parkinson's models are used, rodents and non-human primates tend to be the focus for two reasons: their physiology is closer to human than non-vertebrates and preclinical studies in one or more of these species for a new drug are always necessary before human testing. Currently, there is not one accepted, perfect, or all-encompassing model for Parkinson's disease research, and each may be utilized for its different advantages in modeling the disease.

2.3.1 TOXIN MODELS

A toxin model of Parkinson's disease involves the administration of a substance to the animal that will mainly degenerate the nigrostriatal dopamine system. This loss of dopamine can be unilateral or bilateral and the degree of loss is often assessed through motor behaviour tests, though more recently non-motor behaviour is also measured. Generally speaking, these models do not have obvious α-syn accumulation/Lewy pathology. Their advantage comes from their quick onset and specific destruction of the nigrostriatal dopamine system, which can be evaluated *post mortem* by markers for dopamine and its related metabolites.

In the early 1980s, MPTP (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine), mixed with MPPP (1-methyl-4-phenyl-4-propionoxypiperidine), an analgesic, was sold as a new, synthetic heroin to drug users and several people who injected it developed immediate onset parkinsonism that was responsive to dopamine-replacing drugs (Langston *et al.*, 1983). It was not known at the time since the compound had not been tested in animals, but it was soon discovered that MPTP is the precursor of MPP+ (1-methyl-4-phenylpyridinium), which causes specific nigrostriatal dopamine neuron death (Burns *et al.*, 1983) by a DAT-mediated entry that then inhibits mitochondrial complex I in these cells (Javitch *et al.*, 1985). MPTP is now used as a tool to study Parkinson's disease in animals. A strong advantage of this model is that it produces very similar dopamine neuron loss to that observed in Parkinson's disease (Langston *et al.*, 1983), including selectively destroying the substantia nigra dopamine neurons, with less effect on the VTA dopamine neurons, as well as dopamine terminal loss primarily in the putamen (Snow *et al.*, 2000). The MPTP model is used in mice and primates, however not in rats since interestingly they were found to be resistant to the toxin (Chiueh *et al.*, 1984). Also, since MPTP readily crosses

the blood-brain barrier it can be more easily administered to animals by using intraperitoneal injections (Jackson-Lewis & Przedborski, 2007), and these injections cause a bilateral lesion. The main behavioural outcomes are reduced locomotor behaviour in mice (Jackson-Lewis & Przedborski, 2007), and can also result in more typical motor symptoms of Parkinson's disease in monkeys (Bezard *et al.*, 1997). Non-motor symptoms in non-human primates have also been measured (Schneider & Kovelowski, 1990). However, this model generally does not result in any Lewy pathology in animals (Shimoji *et al.*, 2005; Halliday *et al.*, 2009), though there are reports of it in monkeys (Kowall *et al.*, 2000). In general, the MPTP model has been used more extensively in non-human primates to model Parkinson's disease, and its ability to cross the blood-brain barrier, as well as its similar effect to human Parkinson's patients on the dopaminergic neurons of the nigrostriatal system, make it a useful tool.

Another commonly used toxin model is 6-hydroxydopamine (6-OHDA). 6-OHDA is specific to catecholamine neurons, entering the cell through the DAT or noradrenaline transporter, where it forms reactive oxygen species independently, and also by inhibiting mitochondrial respiratory chain complexes I and IV (Evilsizor *et al.*, 2015). Unlike MPTP, 6-OHDA does not cross the blood-brain barrier and therefore must be administered directly to the brain using stereotaxic injection (Ungerstedt, 1968). Usually the striatum, substantia nigra, or medial forebrain bundle are used to degenerate the nigrostriatal system (Deumens *et al.*, 2002), and the severity of degeneration depends on the injection location (Jeon *et al.*, 1995; Yuan *et al.*, 2005). Similarly to MPTP, it selectively destroys the nigrostriatal dopamine system, with less effect on the VTA (Healy-Stoffel *et al.*, 2014). It is administered unilaterally since bilateral injections show a high mortality rate and severe behavioural deficits in animals (Ferro *et al.*, 2005). 6-OHDA can be used to cause a fairly consistent lesion in rats, though dose and number/location of brain sites may affect the outcome (Penttinen *et al.*, 2016). It has been successfully used in mice, but a higher variation between animals is observed (Iancu *et al.*, 2005). The main behavioural evaluation is also performed by motor tests, typically amphetamine- or apomorphine-induced rotations (Ungerstedt, 1971). Additionally, non-drug behaviour such as cylinder test is used (Schallert *et al.*, 2000). These tests can be used since the unilateral lesion causes dopamine neuron cell death on one side of the brain. Assessing the degree of the lesion, and the ability of a therapy to restore dopamine neurons, is often done by measuring the behavioural outcome on these tests. Typical neuropathology outcome measures are immunostaining for markers of dopamine neurons such as TH and DAT and measurement of dopamine content. However, there is also no evidence of Lewy pathology after 6-OHDA administration. The 6-OHDA model has mostly been used in rats to affect the nigrostriatal dopamine system and results in a clear motor phenotype. In the past, current dopamine replacement therapeutics have been tested in this model and successfully made it to the clinic (Griebel *et al.*, 2000), giving it some validity as a model of Parkinson's disease. Again, as with MPTP, its fast onset and destruction of the nigrostriatal system make it a model that is still used for Parkinson's disease.

There has been research that Parkinson's disease may be caused by environmental toxins (Goldman, 2014), although this is not conclusive, and may not be true for all patients. Rotenone and paraquat have both been used to make Parkinson's toxin models, which are mostly used as a pesticide and herbicide, respectively. These toxins reproduce the dopamine neuron degeneration and motor symptoms similar to MPTP and 6-OHDA in animals (Brooks *et al.*, 1999; Betarbet *et al.*, 2000), although for paraquat it may not be as specific to the dopamine system (Miller, 2007). Additionally, administration of rotenone has been shown to cause high mortality and difficulties in reproducibility (Fleming *et al.*, 2004). The advantage of these toxins however is that they are able to induce Lewy pathology in dopamine neurons (Manning-Bog *et al.*, 2002; Sherer *et al.*, 2003).

Though there are several other exogenous toxins that can be administered to the animal brain to cause destruction of the dopamine system, of some interest are those that affect the ubiquitin-proteasome system. As discussed above, essentially, the ubiquitin-proteasome system clears away or degrades unneeded proteins in the cell, therefore it has become important in Parkinson's disease in relation to α-syn, and some genetic mutations of the disease have been linked to dysfunction in the system (Olanow & McNaught, 2006). One way to affect this is by administration of lactacystin, a proteasomal inhibitor. Lactacystin has been shown to cause dopamine neuron degeneration in rodents after stereotaxic injection to the substantia nigra, including some deficits in motor behaviour (McNaught *et al.*, 2002a; Savolainen *et al.*, 2017) (publication V). Importantly, lactacystin administration has also resulted in buildup of α-syn. This gives the model an advantage over MPTP and 6-OHDA as it sits between a toxin and an α-syn-based model. However, since this buildup of protein is due to a general dysfunction in the proteasome, it is not necessarily specific to α-syn, nor is it specific to dopamine neurons, which gives the model a disadvantage over models that specifically use or target α-syn and the dopaminergic system.

Toxin models reproduce the nigrostriatal dopamine system loss seen in Parkinson's disease; this happens fairly quickly, making it a less time-consuming method for researchers and a useful way to test dopamine-modifying therapies. However, since they mostly lack α-syn pathology this gives them a disadvantage in that they do not consistently recapitulate this hallmark of Parkinson's disease.

2.3.2 GENETIC MODELS

Animal models for the PARK mutations mentioned above have been made in order to recapitulate the genetic forms of the disease. With mice being the model organism of choice for genetic models, these are often in mice. Transgenic overexpression of human wild-type α-syn is perhaps the most obvious of these models. Several lines of mice have been bred using different promotors to drive the transgene as well as using mutant forms of α-syn. For wild-type overexpression, there have been reports of some motor impairment, loss of TH and dopamine, and also accumulation of α-syn (Masliah *et al.*, 2000; Richfield *et al.*, 2002), however these models do not recapitulate the loss of nigrostriatal dopamine neurons. As for the mutated α-

syn transgenic mice, the A30P and A53T are the most commonly used. Both mutations have shown behavioural deficits, decreased levels of dopamine in the striatum, α-syn accumulation, but again no significant neuron loss in the substantia nigra (Gomez-Isla *et al.*, 2003; Ikeda *et al.*, 2009; Oaks *et al.*, 2013). Although this is also variable, since some studies have shown nigral TH+ cell loss without inclusions (Thiruchelvam *et al.*, 2004), and vice versa (Lee *et al.*, 2002). And in the former study, there was only a small difference in TH+ cells that only appeared at 20 months of age compared to wild-type littermates. In general, the outcome seems to depend mostly on which promoter is used and where the gene integrates, making it difficult to compare one mouse line to another (Blesa & Przedborski, 2014).

The other PARK genetic models: LRRK2 (overexpression) (Ramonet *et al.*, 2011), Parkin (KO) (Itier *et al.*, 2003), PINK1 (KO) (Kitada *et al.*, 2007), DJ-1 (KO) (Goldberg *et al.*, 2005), and ATP13A2 (KO) (Schultheis *et al.*, 2013), produce some age-dependent motor impairments, and mild dopamine deficits, though they often do not have α-syn inclusions or strong nigrostriatal loss. In general, there are a number of genetic mutations used for Parkinson's disease animal models, and they may give some hints about disease aetiology, however often their lack of clear nigrostriatal degeneration and α-syn-positive Lewy pathology still limits their use.

2.3.3 ALPHA-SYNUCLEIN VIRAL OVEREXPRESSION MODELS

Overexpressing α-syn directly at the substantia nigra using stereotaxic injections of a virus carrying human α-syn transgene (wild-type or mutant forms) has become a popular animal model for Parkinson's disease. Viral overexpression via lentivirus or AAV vectors are the most often used in order to recapitulate the widespread α-syn pathology in the substantia nigra as well as degenerate the nigrostriatal dopamine system.

Lentiviruses are retroviruses (such as HIV) that are made replication-deficient; the vectors are used to transduce non-dividing cells, making them useful for neurons (Kay *et al.*, 2001). Lentiviral-mediated α-syn overexpression using both the human wild-type and mutant forms, as well as rat α-syn have been studied for their ability to model Parkinson's disease in rodents. In rats, both human wild-type and two missense mutations (A30P, A53T) of α-syn via lentiviral overexpression injected above the substantia nigra led to TH loss in the nigrostriatal areas, the striatum and substantia nigra, as well as α-syn-positive inclusions in the surviving substantia nigra neurons (Lo Bianco *et al.*, 2002). Interestingly, overexpression of rat α-syn also resulted in inclusions but did not show profound cell loss. A study in non-transgenic mice with lentivirus carrying the A30P or wild-type human α-syn, where the animals were followed for one year, showed similar results when injected to the striatum, substantia nigra, and amygdala (Lauwers *et al.*, 2003). Overexpression in these areas led to Lewy pathology in cell bodies, neurites, and the cytoplasm, also in cells other than dopamine neurons. There was a small loss of TH+ cells in the substantia nigra at 10-12 months post-injection. In general, lentiviruses overexpressing human α-syn appear to be effective in disease modelling, however, simultaneously performed studies

with AAV-α-syn showed higher transduction of TH+ cells, and that may be one reason why the model has fallen out of favour (Kirik & Bjorklund, 2003).

Using AAV to overexpress specific genes has been used in Parkinson's disease modeling as well as in treatment (Domanskyi *et al.*, 2015). Due to its ability to safely and efficiently transduce cells long-term (Kaplitt *et al.*, 1994) it has been widely used in animal and human brains (Wu *et al.*, 2006). AAV is a non-replicating virus that often does not result in immune reactions in human cells and is not associated with disease in primates (Chirmule *et al.*, 1999). AAVs are currently being utilized in several clinical trials, including in Parkinson's disease as discussed above. Additionally, its use in Parkinson's disease modeling has been fairly extensive over the last several years. Overexpression of human α-syn using AAV encoding SNCA has been used in rodents and non-human primates, where both the wild-type and mutant forms are utilized. The most common method is to unilaterally inject AAV-α-syn above the substantia nigra where it transduces dopamine neurons of the nigrostriatal tract. This causes a progressive loss of TH and results in the presence of α-syn, and this onset of loss is generally slower and less severe than the toxin 6-OHDA (Decressac *et al.*, 2012a). The loss is accompanied by unilateral motor deficits. This gives the model good validity in modeling Parkinson's, where the sporadic disease has a mostly slow onset and the presence of α-syn. However, these studies use different AAV serotypes and promoters, which may affect cell specificity and degree of transduction (Tenenbaum *et al.*, 2004). Also, wild-type versus mutant α-syn, end points, and outcome measures are often different (reviewed in (Albert *et al.*, 2017), publication I, and summarized in Table 3). These experiments result in TH loss in the substantia nigra pars compacta across species, however it is not always clear if striatal TH, dopamine levels, or motor behaviour are affected.

Table 3. *Summary of AAV-α-syn studies in wild-type rodents that use human forms of α-syn for overexpression to the substantia nigra and measure the typical outcomes for the nigrostriatal system and related behaviour compared to a control. Modified from (Albert et al., 2017).*

Strain	Insert	Serotype/ Promoter	Duration	STR TH fibre dens.	SNpc TH+ cells	STR DA	Behav.	Ref.
C57BL/ 6 mice	WT A53T	AAV2/7 / CMV (w/ WPRE)	8 weeks	N/A	↓	N/A	WT: motor deficits	(Oliveras-Salva *et al.*, 2013)
C57BL/ 6 mice	WT	rAAV / CMV (w/ WPRE, IRES)	24 weeks	N/A	↓	N/A	N/A	(St Martin *et al.*, 2007)
C57BL/ 6 mice	A53T	AAV1/2 / CBA (w/ CMV, WPRE, bGH-polyA)	10 weeks	↓	↓	↓	Motor deficits	(Ip *et al.*, 2017)

C57BL/6 mice	A53T	rAAV (w/ WPRE)	7 weeks	↔	↔	↔	N/A	(Dong *et al.*, 2002)
C57BL/6 mice	WT	AAV2 / CBA (w/ WPRE, IRES)	24 weeks	N/A	↓	N/A	N/A	(Thome *et al.*, 2015)
C57BL/6 mice	WT	rAAV2/1	12 weeks	↓	↓	↓	Motor deficits	(Song *et al.*, 2015)
C57BL/6 mice	WT	AAV2 / CBA	8 weeks	↓	↔	↓	↓	(Svarcbahs *et al.*, 2016)
SD rats	WT	rAAV / CBA (w/ CMV elements)	27 weeks	↓	↓	↓	No motor deficits*	(Kirik *et al.*, 2002)
SD rats	WT	AAV6 / CBA (w/ and w/out WPRE)	16 weeks	↓ WPRE	↓ WPRE	↓ WPRE	Motor deficits w/ WPRE	(Decressac *et al.*, 2012b)
SD rats	WT	AAV2/5 / CBA/ CMV	8 weeks	N/A	↓	N/A	Some motor and non-motor deficits	(Gombash *et al.*, 2013)
Fisher 344 rats	WT	AAV9 / CBA	12 weeks	N/A	↓	N/A	Motor deficits	(Shahaduzzaman *et al.*, 2015)
SD rats	WT	rAAV / CMV	13 weeks	N/A	↓	↔	No motor deficits	(Yamada *et al.*, 2004)
SD rats	WT S129A S129D	AAV2/5 / CMV/CBA	15 weeks	↓	↓	N/A	Some motor deficit	(Febbraro *et al.*, 2013)
SD rats	A53T	AAV1/2 / CBA/CMV (w/ WPRE, bGH-polyA)	6 weeks	↓	↓	↓	Some motor deficit	(Koprich *et al.*, 2011)

SD rats	WT A30P	AAV2/6 / CMV (w/ WPRE)	16 weeks	↓	↓	↓	Some motor deficit	(Gaugler *et al.*, 2012)
Wistar rats	WT A53T	AAV2/7 / CMV/ synapsin	29 days	N/A	A53T: ↓	A53T: ↓	A53T: Some motor deficit	(Van der Perren *et al.*, 2015)
SD rats	WT	AAV5 / human U6	8 weeks	N/A	↓	↓	No motor deficit	(Gully *et al.*, 2016)
SD rats	WT	AAV5 / CMV	8 weeks	↓	↓	N/A	N/A	(Landeck *et al.*, 2016)
SD rats	WT	AAV2/6 / CMV/ synapsin (w/ WPRE)	5 weeks	↓	↓	↓	Motor deficits	(Decressac *et al.*, 2013)
SD rats	A53T	AAV1/2 / CBA/CMV (w/ WPRE, bGH-polyA)	6 weeks	↓	↓	↓	Motor deficits	(He *et al.*, 2016)
SD rats	WT	AAV6 / synapsin (w/ WPRE)	8 weeks	↓	↓	N/A	Some motor and non-motor deficits	(Caudal *et al.*, 2015)
SD rats	A53T	AAV2 / CMV (w/ CBA, WPRE)	8 weeks	↓	N/A	N/A	N/A	(Arawaka *et al.*, 2014)
Wistar rats	WT A53T	rAAV / CBA/CMV (w/ WPRE)	12 weeks	↓ no quant.	↓ no quant.	N/A	Motor deficit	(Lu *et al.*, 2015)
SD rats	WT S129A S129D	AAV2/8 / CBA (w/ WPRE)	6 weeks	↔	↓	↔	N/A	(McFarland *et al.*, 2009)
Fisher 344 rats	WT	AAV9 / CBA/CMV (WPRE, bGH-polyA)	16 weeks	N/A	↓	N/A	N/A	(Pabon *et al.*, 2012)

SD rats	WT	AAV2/5 / CMV	18 weeks	↔	↔	N/A	N/A	(Moloney *et al.*, 2014)
SD rats	WT	AAV2/5 / pDG-5 promoter	24 weeks	↓	↓	N/A	Motor deficits	(Mulcahy *et al.*, 2012)
SD rats	WT	AAV2/6 / synapsin (w/ WPRE) bilateral injections	40 weeks	↔	↓	N/A	Motor deficits	(Crowley *et al.*, 2018)
Rats	WT S129A S129D	rAAV / CBA (w/ CMV elements)	26 weeks	N/A	↓	↓WT S129A	N/A	(Gorbatyuk *et al.*, 2008)
Rats	WT	AAV5	8 weeks	N/A	↓	↓	Motor deficits	(Gorbatyuk *et al.*, 2012)
SD rats	WT	rAAV2/5 / CBA	15 weeks	N/A	↓	N/A	N/A	(Sanchez-Guajardo *et al.*, 2010)
SD rats	A53T	AAV2 / synapsin	24 weeks	N/A	↓	↓	N/A	(Chung *et al.*, 2009)
SD rats	WT	rAAV2/5 / CBA/CMV (w/ WPRE)	13 weeks	N/A	↔	N/A	Motor deficits	(Andersen *et al.*, 2018)

N/A refers to the fact that an outcome measure was not included in the article; ↓ A decrease in the parameter was shown; ↔ No change in the parameter was shown; * With TH enzyme blocker on stepping test; 'Rats' indicates no strain specified in article; STR: striatum; SNpc: substantia nigra pars compacta; SD: Sprague Dawley; TH: tyrosine hydroxylase; DA: dopamine; CBA: chicken β-actin; CMV: cytomegalovirus; WT: wild-type; WPRE: woodchuck hepatitis virus posttranscriptional regulatory element; IRES: internal ribosome entry site; bGH-polyA: bovine growth hormone polyadenylation sequence.

The AAV overexpression model has been shown to be successful in its recapitulation of α-syn expression in the nigrostriatal tract accompanied by TH loss and motor behaviour deficits, but there are also serious flaws to it. A recent study concluded that using AAV encoding human α-syn in rats is not an ideal model for sporadic Parkinson's disease (Su *et al.*, 2017). Since in the AAV model, α-syn mRNA is constitutively increased (Decressac *et al.*, 2012b), however in Parkinson's disease SNCA mRNA is actually decreased (Kingsbury *et al.*, 2004). The above-mentioned study confirmed that SNCA is upregulated in the AAV-α-syn model in rats (Su *et al.*, 2017). Additionally,

there have been concerns with controls (publication I). It has been shown that when AAV-eGFP is expressed at the same titer as AAV-α-syn, it can also cause loss of TH (Landeck *et al.*, 2016; Andersen *et al.*, 2018), and that AAV itself may downregulate TH (publication II).

While the face validity of AAV-α-syn is sound since it appears to be imitating the disease well, it is pertinent to ask whether this is the most accurate model of sporadic Parkinson's disease – in other words does it have construct validity. The need for an appropriate control and problems with the model also bring up questions about its usefulness in therapy testing.

2.3.4 PREFORMED ALPHA-SYNUCLEIN FIBRILS MODEL

Overexpression of α-syn via viral vector may be useful, however α-syn is expressed at high levels, transduces the neurons, and causes rapid cell death due to protein buildup, which does not necessarily mimic sporadic Parkinson's disease. Recently, a new model was developed and has become popular over the last few years. The preformed α-syn fibrils (PFF) model of Parkinson's disease mimics the way α-syn acts like a prion protein as well as the formation of Lewy bodies: when it is injected stereotaxically or applied to culture it spreads from one neuron to another by recruiting endogenous α-syn that builds up in the cell body and neurites.

As mentioned above, the fibrillar form of α-syn is considered an insoluble form of the protein that relates to aggregation and formation of Lewy bodies. Since there is clear Lewy pathology in human brains with Parkinson's disease, *in vitro* and *in vivo* models using fibrils to mimic this were developed. Luk and others (2009) demonstrated that purified human wild-type α-syn fibrils will seed endogenous α-syn and form Lewy body-like inclusions that contain ubiquitin, are insoluble, and are pS129-positive (Luk *et al.*, 2009), thus making them similar to Lewy bodies in human disease. A further study using primary neuron culture demonstrated that the Lewy bodies formed by the fibrils propagate in the cells and consequently lead to neuronal dysfunction and death (Volpicelli-Daley *et al.*, 2011).

With their ability to form Lewy pathology and cause cell death *in vitro*, the PFFs have now been used extensively in rodents. From a later paper by Luk and others came one of the first studies in wild-type mice (Luk *et al.*, 2012). The authors demonstrated that injection of mouse PFFs into the striatum of the mice resulted in clear α-syn-positive inclusions throughout the ipsilateral striatum, cortex, olfactory bulb, amygdala, and importantly the substantia nigra pars compacta. This was accompanied by reduced dopamine content at three months post-injection, TH loss in the striatum and substantia nigra at six months, as well as behavioural deficits. Interestingly, these deficits appear only after three to six months after injection, demonstrating that this is a much slower process than either viral vectors or toxin models. Although, perhaps unlike in Parkinson's disease in humans, α-syn is seeded and spread much more quickly in the model in mice (Okuzumi *et al.*, 2018). Other groups have shown similar results using PFFs in rodents and primates. In rodents, PFFs have been injected to several different areas. Injection of mouse PFFs to the striatum of both mice and rats results in robust pS129 pathology in the cortex, amygdala, and substantia nigra pars

compacta (Abdelmotilib *et al.*, 2017). In both species the amount of α-syn pathology correlated to loss of TH+ cells in the substantia nigra pars compacta. Interestingly, injection of pS129 PFFs into the mouse striatum resulted in TH cell loss in the substantia nigra pars compacta and some motor impairment already at two months, compared to the wild-type PFFs (Karampetsou *et al.*, 2017), but at the moment this has not been explored further. In a study that also injected PFFs into the striatum of mice, the authors showed that there was dendritic spine loss in the cortex (Blumenstock *et al.*, 2017). Although the same was not the case when PFFs were injected into the hippocampus of mice, where pS129-positive inclusions formed in hippocampal neuronal connections but there was no cell loss or memory deficits (Nouraei *et al.*, 2018). Another study where PFFs were injected into the olfactory bulb in order to create a prodromal model of Parkinson's disease showed inclusions in connecting brain areas and that there were deficits in the olfactory system measured through behavioural tests; this occurred progressively over the 12 months of the study (Rey *et al.*, 2016), but leveled off, in other words there was no further spreading or increase in severity of pathology, at 23 months (Rey *et al.*, 2018). Other than the olfactory bulb, it is thought that α-syn pathology may spread from the gastrointestinal tract to the central nervous system via the vagus nerve, thus also explaining the prodromal gastrointestinal symptoms observed in Parkinson's disease. A study in mice applied PFFs to the gastric wall and found phosphorylated α-syn/Lewy body pathology in the dorsal nucleus of the vagus nerve, however this did not propagate further and was not specific to any particular cell type, as would be hypothesized with Braak staging pathology (Uemura *et al.*, 2018). However, a similar study in rats and non-human primates found gastrointestinal deficits in the rat after PFFs were injected to the descending colon, as well as Lewy body-like inclusions in the enteric nervous system and brain stem (Manfredsson *et al.*, 2018). For the non-human primate, injection of PFFs into the colon and stomach also resulted in enteric nervous system pathology. When rats are used for PFF studies, similar results have been obtained as for mice with striatal injections of mouse PFFs. Lewy body-like inclusions were found in cortex, amygdala, and substantia nigra pars compacta, and this resulted in degeneration in the dopamine system at six months post-injection, however no motor deficits were observed (Paumier *et al.*, 2015). Another study in rats had similar results, and also demonstrated microglial activation, related to an inflammatory reaction in the brain (Duffy *et al.*, 2018). Additionally, injection of mouse PFFs to the marmoset putamen resulted in pS129-positive inclusions and loss of TH+ cells in the substantia nigra pars compacta on the ipsilateral side already at three months after injection (Shimozawa *et al.*, 2017).

The PFF model appears to be relatively successful across species and consistently results in seeding of endogenous α-syn in the connected areas, although cell death and behavioural deficits are not always present. This may depend on the species of purified α-syn used (ex. human versus mouse), since it was shown that seeding may be more efficient in the matching species, in other words α-syn that was more homologous to mouse α-syn was more effective in mice (Luk *et al.*, 2016). Additionally, the lack of severe nigrostriatal dopamine degeneration has been overcome by simultaneously

overexpressing α-syn via AAV with PFF injection (Thakur *et al.*, 2017). In general, as with any animal model that adds exogenous material, severity may depend on how much is added and the duration of the experiment.

2.4 CEREBRAL DOPAMINE NEUROTROPHIC FACTOR (CDNF)

Cerebral dopamine neurotrophic factor (CDNF) was first characterized in 2007 (Lindholm *et al.*, 2007). Mature human CDNF is 161 amino acids and is highly conserved in vertebrates. Although its mechanism of action is dissimilar to other neurotrophic factors, it has been shown to be effective in animal models of disease, most importantly Parkinson's.

2.4.1 CDNF AND RELATED FACTOR MANF

Human mesencephalic astrocyte-derived neurotrophic factor (MANF) shares 59% amino acid identity with human CDNF (Lindholm *et al.*, 2007; Lindholm & Saarma, 2010), and these two factors form an evolutionarily conserved group of proteins. Their similar structure may be related to both lipid or membrane binding, as well as regulation of ER stress (Parkash *et al.*, 2009), which gives hints about how these proteins function. Specifically, the N-terminal domain is a saposin-like domain which is known to interact with lipids, and the C-terminal with its single cysteine bridge is similar to other proteins that function at the ER (Ellgaard & Ruddock, 2005). Furthermore, at the end of the proteins' C-terminus are a KTEL (CDNF) and RTDL (MANF) sequence which are known as ER retention signals. And in fact, *in vitro* deletion of the RTDL sequence in MANF prevented its localization to the ER (Glembotski *et al.*, 2012; Matlik *et al.*, 2015), and in another study increases its secretion as well (Henderson *et al.*, 2013). Interestingly, in the same study, the RTDL sequence was not required for *in vivo* protection of neurons but seemed to be dependent on the CXXC part of the C-terminal domain. It has also been demonstrated that CDNF may function in a similar way with its KTEL sequence (Norisada *et al.*, 2016). MANF expression has been shown to increase after thapsigargin, tunicamycin, and lactacystin treatment of cells (Apostolou *et al.*, 2008), compounds known to activate the unfolded protein response (UPR). Additionally, its cellular secretion in response to thapsigargin occurs only when ER calcium is depleted (Henderson *et al.*, 2014).

Both MANF and CDNF (human or rodent) are expressed in the central and peripheral nervous systems of adults. Interestingly, MANF has been detected in human blood serum at a level of 7 ng/mL (Galli *et al.*, 2016). While levels of CDNF mRNA and protein have been shown to be generally lower than MANF, they show a partially overlapping expression pattern (Lindholm *et al.*, 2007). CDNF transcript has been shown to be present in the human brain, and in adult mice using a CDNF-specific antibody it was detected in the cerebral cortex, hippocampus, locus coeruleus, and the Purkinje cells of the cerebellum. In the periphery, human CDNF is at its highest levels in skeletal muscle, heart, lung, and testis compared to other tissues.

A model of MANF KO has been characterized and it was found that these mice develop type 1 diabetes due to loss of pancreatic β cells (Lindahl *et al.*, 2014), which was caused by increased and constitutive activation of the UPR pathway (Danilova *et al.*, 2019). However, characterization of CDNF KO mice has shown that they are viable and fertile, although they have some defects in the enteric and midbrain dopamine neurons (Lindahl et al., manuscript).

MANF, and CDNF in particular, are unlike other classical neurotrophic factors such as GDNF. GDNF, for example, will, upon secretion, bind to its co-receptor GFRα1 to activate transmembrane tyrosine kinase receptor Ret and promote neuronal growth and survival (Airaksinen & Saarma, 2002). By contrast, CDNF's mode of action is still unclear. Both factors are functioning intracellularly and CDNF seems not to be secreted from neurons, even after robust overexpression via lentivirus (Matlik, 2017). Their N-terminal and C-terminal sequences connote dual modes of action: the N-terminal motif seems to be important for lipid binding and the C-terminus exerts effects for localization at the ER. Though similarly to classical neurotrophic factors, CDNF has been shown to protect neurons *in vitro* (Arancibia *et al.*, 2018) and *in vivo* when a stressor or lesion is applied. Additionally, these proteins are likely functioning differently when given exogenously, as opposed to their endogenous actions. There is evidence of this from when CDNF was exogenously applied by stereotaxic injection to the brain and it was shown to be efficiently taken up by the neurons (Matlik *et al.*, 2017). However, further research is needed to fully comprehend how CDNF, and MANF, are functioning in the central nervous system.

2.4.2 CDNF IN PARKINSON'S DISEASE

When CDNF was first characterized, it was shown to be neuroprotective in the 6-OHDA model of Parkinson's disease (Lindholm *et al.*, 2007). When CDNF was injected stereotaxically to the striatum four weeks after 6-OHDA in rats, it resulted in significantly fewer amphetamine-induced ipsilateral rotations and protected the TH+ cells in the substantia nigra pars compacta compared to vehicle. Since then, it has been shown to not only be protective in Parkinson's disease models, but also in other rodent disease models. CDNF enhanced long-term memory in wild-type mice and in a transgenic model of Alzheimer's disease, however it did not increase neurogenesis or decrease amyloid-β accumulation (Kemppainen *et al.*, 2015). It was also shown to be anti-inflammatory when given via bone marrow-derived mesenchymal stem cells that overexpress CDNF to a rat model of spinal cord injury where it reduced expression of cytokines, protected the cells from injury, and resulted in improved locomotor behaviour (Zhao *et al.*, 2016).

Since the initial study showed CDNF to be neuroprotective in a 6-OHDA model, it has been studied the most for Parkinson's disease. In a mouse model using MPTP, CDNF was given bilaterally to the striatum before or after MPTP which resulted in improved locomotor activity, as well as protection of TH fibres in the striatum and TH+ cells in the substantia nigra pars compacta (Airavaara *et al.*, 2012). In another 6-OHDA experiment,

CDNF was given continuously over a two-week period to the striatum after the toxin injection, which also resulted in recovery on amphetamine-induced rotations, as well as protecting against the loss of TH fibres in the striatum and TH+ cells in the substantia nigra pars compacta (Voutilainen *et al.*, 2011). Thus, CDNF seems to be protective over long administration periods, as opposed to GDNF, where sustained overexpression does not necessarily result in increased improvement over single injection (Penttinen *et al.*, 2018b). A recent study repeated the experiment similarly to Lindholm *et al*. 2007, but with a lower dose of CDNF (Voutilainen *et al.*, 2017), however at the low doses used CDNF did not reach significance compared to vehicle. Another study administered CDNF to the substantia nigra one week after a 6-OHDA lesion of the medial forebrain bundle but did not show any positive effects, however interestingly, when CDNF was given to the substantia nigra four weeks after the lesion, there was some functional recovery on apomorphine-induced rotations (Huotarinen *et al.*, 2018). This study also used CDNF injection to the substantia nigra in combination with deep brain stimulation of the STN and showed a positive effect on apomorphine-induced rotations and cylinder test compared to CDNF or stimulation alone. However, the efficacy of CDNF may depend on lesion size and progression as well as on the CDNF administration paradigm. Our unpublished results show that there is a clear functional recovery on amphetamine-induced rotations at eight weeks when CDNF was given as a single injection to the striatum, substantia nigra, or both, two weeks after a severe 6-OHDA lesion, but there was no protection of TH fibres in the striatum or TH+ cells in the substantia nigra at twelve weeks when the experiment was ended (unpublished results). Therefore, caution in experimental setup is crucial, and naturally the degree of the lesion will strongly affect the outcome.

The above studies use the exogenous human CDNF protein alone, but AAV carrying human CDNF has also been used somewhat successfully in the 6-OHDA model. Rats received AAV2-CDNF two weeks before 6-OHDA and while this reduced the number of amphetamine-induced rotations, it only showed some protection of striatal TH fibres and nigral TH+ cells at the highest titer (Back *et al.*, 2013a). This could be due to the spreading of the protein in the striatum with AAV: there was very minimal detection of the protein in the striatum and substantia nigra with immunohistochemistry, but Voutilainen and others (2011) and Mätlik and others (2017) show that CDNF protein alone is spreading robustly in the striatum at two to six hours, and that it is detected in the substantia nigra (Voutilainen *et al.*, 2011; Matlik *et al.*, 2017). We have also observed similar results with the CDNF protein by itself after nigral injection (publication IV). However, despite this, another study has shown significant positive results with AAV2-CDNF (Ren *et al.*, 2013), and AAV8-CDNF also showed positive effects on TH, but only in a mild lesion paradigm (Wang *et al.*, 2017). Additionally, a study using lentiviral CDNF showed less efficacy after striatal injection when given at the same time as 6-OHDA, but did show some functional effect on behaviour after nigral injection (Cordero-Llana *et al.*, 2015). However, this study did not show any CDNF expression data and due to the composition of the expression vector it is difficult to interpret where it localizes in the brain, and therefore it may not be optimally exerting its effects. A study using a transiently transfected CDNF plasmid carried by a neurotensin-polyplex was injected to

the substantia nigra of rats after a striatal 6-OHDA lesion and resulted in reduced numbers of activated glial cells in the substantia nigra pars compacta (Nadella *et al.*, 2014). CDNF has also been tested in monkeys, where it showed an increase in DAT activity in the striatum after a 6-OHDA lesion (Garea-Rodriguez *et al.*, 2016). Importantly, it is currently in clinical trials for Parkinson's disease in Sweden (Herantis Pharma, 2017b) and Finland (Herantis Pharma, 2017a). A brief summary of the CDNF animal studies in Parkinson's models is shown in Table 4.

Table 4. *Summary of studies using CDNF in animal models of Parkinson's disease toxin models.*

Experiment	*Main result*	*Reference*
CDNF protein (10µg) unilateral injection to STR of rats	Positive effects on behaviour and TH	(Lindholm *et al.*, 2007)
CDNF protein (10µg) bilateral injection to STR of mice	Positive effects on behaviour and TH	(Airavaara *et al.*, 2012)
CDNF protein chronic infusion to STR of rats	Positive effects on behaviour and TH	(Voutilainen *et al.*, 2011)
CDNF protein (1-5µg) unilateral injection to STR of rats	No significant effects on behaviour and TH compared to vehicle	(Voutilainen *et al.*, 2017)
CDNF protein (10µg) unilateral injection to SN of rats	Positive behaviour effect; no significant effect on TH compared to vehicle	(Huotarinen *et al.*, 2018)
AAV2-CDNF unilateral injection to STR of rats	Positive effects on behaviour and TH	(Back *et al.*, 2013a)
AAV2-CDNF unilateral injection to STR of rats	Positive effects on behaviour and TH	(Ren *et al.*, 2013)
AAV8-CDNF unilateral injection to STR of rats	Positive effects on behaviour and TH	(Wang *et al.*, 2017)
Lentiviral CDNF unilaterally to STR or SN of rats	No significant effects on behaviour or TH with STR injection; positive effects on behaviour with SN injection	(Cordero-Llana *et al.*, 2015)
CDNF plasmid to SN of rats	Positive effects on inflammatory cells	(Nadella *et al.*, 2014)
CDNF protein chronic infusion to STR of monkeys	Increase in DAT activity	(Garea-Rodriguez *et al.*, 2016)

While CDNF has shown clear efficacy in toxin models, we only have some evidence of how it fares in α-syn-based models (manuscript, publication III). A published *in vitro* study showed that application of an excessive amount of CDNF to neuronal culture treated with a large amount of PFFs one hour before resulted in protection of the cells (Latge *et al.*, 2015). This is interesting, and our results give hints towards a direct interaction between CDNF and α-syn (manuscript, publication III), as well as some positive *in vitro* and *in vivo* effects. However, further experiments are certainly needed to elucidate the mechanism and understand the full effect of CDNF on α-syn in Parkinson's disease models.

3 AIMS

The overall aim of this thesis was to model α-synuclein-based Parkinson's disease in rodents in order to test cerebral dopamine neurotrophic factor (CDNF) as a therapy. CDNF has been effective in neurotoxin models, and it is currently in clinical trials for Parkinson's disease patients. Therefore, it became pertinent to test it in α-synuclein models.

The specific aims were:

1. Model Parkinson's disease in rats using adeno-associated virus (AAV) to overexpress human α-synuclein. AAV-α-synuclein models have been shown to successfully recapitulate typical features of toxin models: nigrostriatal neurodegeneration and motor behavioural deficits, but with the presence of α-synuclein. Therefore, we wanted to validate this as a model for testing new therapies.

2. Model Parkinson's disease in rodents using preformed fibrils to seed endogenous α-synuclein in the brain in order to test CDNF's effects on the model. The preformed α-synuclein model has been shown to recreate the spreading of α-synuclein from cell to cell *in vitro* and *in vivo*, accompanied by deficits in motor behaviour and loss of nigrostriatal dopamine neurons in mice and rats. It is considered to be a model of Lewy pathology and progressive spreading of pathogenic α-synuclein. We therefore set up this model to test CDNF in.

3. Characterize the effects of nigral administration of CDNF *in vivo*. CDNF is currently in clinical trials for Parkinson's disease and the striatal administration has been characterized, therefore we wanted to characterize its effects when stereotaxically injected to the substantia nigra of naïve rats.

4. Model an α-synuclein-based Parkinson's disease phenotype in mice using lactacystin. Injecting the proteasomal inhibitor lactacystin to the substantia nigra of mice has resulted in protein buildup in the nigrostriatal tract, including α-synuclein, as well as behavioural deficits and loss of dopamine neurons. We wanted to further characterize and validate this model as another way of modeling α-synuclein-related Parkinson's animal models.

4 MATERIALS AND METHODS

Table 5. *Collection of the main methods used in the studies.*

Method	Study
Preparation of injection material	
6-OHDA preparation	II
CDNF preparation	III, IV
Iodination of proteins	IV
Preformed fibrils preparation	III
Viral vector preparation	II
Stereotaxic injections	II, III, IV, V
Behaviour	
Amphetamine-induced rotations	II
Coat hanger test	III
Cylinder test	II, III, V
Locomotor activity	V
Rotarod	III
Tissue collection and processing	II, III, IV, V
Biochemical assays	
High-performance liquid chromatography (HPLC)	II, III, V
Immunohistochemistry	II, III, IV, V
Immunofluorescence	III, IV, V
Imaging and analysis	
Confocal microscopy	IV, V
Counting of phosphorylated alpha-synuclein inclusions	III
Counting of TH+ cells	II, V
Optical density measurement	II, III, V

4.1 METHODOLOGICAL CONSIDERATIONS

4.1.1 ANIMAL STRAINS

The following strains were used in the experiments:

- C57BL/6JRccHsd mice (male)
- C57Bl/6JRj mice (male)
- C57BL/6JOlaHsd mice (male)
- CDNF KO (male)
- Hsd:HanWistar rats (male)
- Hsd:Sprague Dawley rats (male and female)

It is well-known in the scientific community that strain can potentially affect the results of animal experiments. Effects can range from very clear, such as the fact that MPTP, a toxin used to model Parkinson's disease due to its mechanism in humans, is most effective in a certain strain of mice (Riachi & Harik, 1988), to more complex, for example the observations that mouse strains have varying levels of anxiety and aggression (Parmigiani *et al.*, 1999), and that behaviour can even be different throughout an animal's lifespan (Shoji *et al.*, 2016). Strain needs to be factored into experiment planning, and can have long reaching effects, particularly when considering inbred strains as well as their use in breeding genetically modified animals. Though we have

used genetic models minimally in the current studies, strain is an important aspect here, particularly in regard to the α-syn models.

While inbred strains of animals are considered to be isogenetic, it is possible that expression levels of certain proteins may change over time due to continuous breeding (Davis *et al.*, 2012). This can be particularly problematic in the long term, resulting in higher variability between animals, experiments, and labs. Although one could take a more optimistic perspective and consider that if your model or treatment works from experiment to experiment, and similarly in another lab, then this would give increased clinical relevance to it since humans are not all the same genotype. However, it is nonetheless important to have knowledge of such strain differences. An important example from the current studies is the C57BL/6JOlaHsd strain of mice, where it was discovered that these mice carry a mutation for the α-syn gene (Specht & Schoepfer, 2001). This essentially makes these α-syn KO animals. As long as researchers are aware of this, these mice can be used as controls in experiments, as we have done (publication III), but if the researcher is not aware, this can be a significant waste of time, effort, and money. This is especially true in general for behavioural neuroscience experiments, and in this case α-syn-based experiments.

In contrast to measurable changes at the gene level, animal strains may differ in their behaviour as mentioned above. This is more difficult to quantify with certainty, and also may change between labs and animal suppliers. However, in the current studies we found differences in non-drug induced motor behaviour between rat strains. In publication II we observed significantly more rearings with the Sprague Dawley rats compared to the male Wistar rats (publication II, Figure 1d). Therefore, we used this strain for future studies since with a higher number of rearings per rat we can obtain more accurate results. This demonstrates that even a supposedly straightforward factor such as the natural behaviour of a rodent standing on its hindlegs can be affected by strain.

Another important factor in animal studies is the sex of the animal. Male mammals are most often used by researchers due to concerns about hormonal cycles affecting inter-subject differences (Beery & Zucker, 2011). However, several studies have revealed sex differences in neurodegenerative diseases (Tierney *et al.*, 2017), and it is therefore important to use both sexes in studies or to consider that studies in, for example, male rodents, do not extrapolate to female rodents. Just as we must consider the jump from rodents to primates to humans in research, we must consider sex differences as well. In these studies, we have also used mostly male rodents, except in publication II where we used female Sprague-Dawley rats fairly exclusively, and this is mentioned. However, any potential sex differences were not examined. This is something that could be considered in future studies, particularly since there are reported gender differences between men and women in Parkinson's disease, where women had higher striatal dopamine, lower incidence of the disease, as well as later age of onset than men (Haaxma *et al.*, 2007), indicating sex as a fairly significant factor.

4.1.2 INTRACRANIAL MICROINJECTIONS

An aspect of stereotaxic injections that is often not discussed in the literature is which injection paradigm to use. When considering intracranial injections of any kind, we tend to focus on the target, but what receives less attention is the fact that we are placing a needle from the top of the skull deep into the brain. The needle has the opportunity to wreak havoc in the surrounding tissue that may cause problems related to unspecific damage of neurons and arteries, as well as inflammation. Therefore, any way to mitigate, or at least be aware, of these problems is useful.

While location of the injection is usually an obvious consideration, needle type, volume, and flow rate are factors that can greatly affect the outcome of an injection. Extensive testing may be needed to find the optimal paradigm for the substance you are injecting. For example, needle gauge and number of injection locations have been shown to affect the outcome in a 6-OHDA model in rats (Penttinen *et al.*, 2016). A potential confounding factor here is that wider steel needles may cause more unspecific damage to the surrounding area of the brain than thinner needles, and in the aforementioned study it was calculated that the larger needle diameter could cause a mechanical lesion equal to 5.1% of the total striatal volume. In the case of publication II, we used glass capillaries to inject the AAVs which have been shown to cause less mechanical damage than steel needles (Gonzalez-Perez *et al.*, 2010), and in publication III we used thinner steel 33G needles for injections to reduce mechanical damage. Concerning flow rate and number of injection locations, we tested this thoroughly in publication II. We found that injecting the AAVs to one site above the substantia nigra at a volume of 4 µl with a flow rate of 0.1 µl/min resulted in widespread expression of the transgene (publication II, Figure 1a), whereas when we injected the AAVs to two sites (2 µl/site) and a flow rate of 0.5 µl/min, this resulted in specific midbrain expression (publication II, Figure 1b-c). Therefore, it is clear that injection paradigms can affect spread and expression of the substance of interest, as well as considerations of mechanical damage that may result in further unspecific destruction in the brain. These factors need to be considered based on what one aims to achieve.

4.1.3 POSITIVE AND NEGATIVE CONTROLS IN DISEASE MODELING

The impact of having proper positive and negative controls cannot be overstated. While it is more common in the literature to have both positive and negative controls in therapeutic drug testing so that it is clear that the drug of interest is having the desired effect, a proper control to compare in disease modeling is less discussed.

In this case, disease modeling refers to injecting a protein or substance of interest in order to recapitulate factors that constitute a neurodegenerative disease in an animal, thus making an animal model of disease to either further understand disease aetiology or to test a therapy. In this thesis, we have used a 6-OHDA toxin model, the lactacystin model, an AAV-α-syn model, and the α-syn PFF model. In these models, a positive control may be less necessary since we are trying to characterize the effects of the injection material in the animals, and therefore there may not be an appropriate comparison. However, when establishing a new model it may be useful to

compare the outcome to another similar model that is the current standard in the field. For example, (Decressac *et al.*, 2012a) showed a side by side comparison of 6-OHDA versus AAV-α-syn when the latter model was first becoming established. Using 6-OHDA as a positive control for comparison allowed the authors to show the differences and potential advantages of AAV-α-syn over the 6-OHDA toxin. Additionally, we have used 6-OHDA similarly as a positive control in publication II, where we wanted to show the clear deficits in cylinder behaviour and how it correlates to loss of striatal TH in the 6-OHDA model, however the same was not the case with AAV-α-syn; there was essentially no correlation between cylinder behaviour and TH loss in the striatum in the AAV model (publication II, Figure 2f-g). These comparisons can be important for other researchers to further understand the model, and that it may not be working in the same way as already established models for Parkinson's disease. This type of comparison to a positive control can save researchers time and effort in how they measure outcomes.

In disease modeling, negative controls are a necessity. One has to be sure that their protein or substance of interest is specifically causing the outcome, or it may not constitute a good disease model. In toxin models such as 6-OHDA and lactacystin, the negative control is often phosphate buffered saline (PBS), saline, artificial cerebral spinal fluid (aCSF), or a sham operation. These can be useful, with the idea being that they are not causing any damage on their own and also controlling for the injection. However, a different control for 6-OHDA could be a substance that would cause less specific neuronal damage, instead of specifically degenerating the dopaminergic system so it would be clear that the outcome measures in the model are useful and correct. Additionally, in the case of 6-OHDA, where a unilateral injection is used, the uninjected side acts as an internal control since there should be a lower degree of neuronal loss there. For lactacystin, since it is an unspecific proteasomal inhibitor, potentially better negative controls may be small molecules that would bind to the proteasome but would either activate it or show no inhibition (Leestemaker *et al.*, 2017).

For models of protein overexpression, such as the AAV-α-syn model, negative controls to account for unspecific damage due to a protein being expressed at high levels are absolutely paramount. Since, as mentioned, expressing eGFP via AAV at the same titer as AAV-α-syn can cause similar loss of the substantia nigra pars compacta TH+ neurons (Landeck *et al.*, 2016; Andersen *et al.*, 2018). We have obtained similar results in our study (publication II), thus demonstrating the importance of having proper controls. Indeed, it would be necessary to have a control to compare to AAV-α-syn that would result in α-syn-specific effects, or the model would be less useful when considering it as a model of Parkinson's disease, and would rather be more similar to a toxin model. In the literature, there are instances where injections are not compared to a control at all, or compared to an empty vector, but a control that expresses a protein that functions similarly to α-syn would be the most useful, so that it is clear that the effects are α-syn-specific.

In the α-syn PFF model, the control used is often the monomeric form of α-syn (Luk *et al.*, 2012), in that this should not seed endogenous α-syn. We have used PBS to control for the effect of the injection and have tested the PFFs in KO animals, where they do not show any phosphorylated α-syn

immunostaining (publication III). These are both good practices, however, use of fibrils of a different pathogenic protein in wild-type mice would also be a useful negative control. There are several options for a negative control to use with α-syn PFFs: tau fibrils (Nonaka *et al.*, 2010), amyloid-β fibrils (Brahic *et al.*, 2016), prion protein (Barron *et al.*, 2016), SOD1 (superoxide dismutase 1) fibrils (Chia *et al.*, 2010), and huntingtin fibrils (Pieri *et al.*, 2012). Caution would need to be taken with amyloid-β fibrils since there is the NAC region of α-syn, which could result in similarities in outcome due to cross reactivity. However, tau fibrils would be a good candidate since presumably the behavioural and histological outcome would be different to α-syn PFFs (Sanders *et al.*, 2014). Additionally, huntingtin fibrils could be another useful control since they have been shown to be taken up and seeded by a different mechanism than tau and α-syn fibrils (Holmes *et al.*, 2013).

4.2 PREFORMED ALPHA-SYNUCLEIN FIBRILS SELECTED METHODS

4.2.1 ANIMALS

2-3 month-old male mice (C57BL/6JRccHsd), approximately 1-year-old male mice (C57Bl/6JRj), young male rats (Hsd:HanWistar and Sprague-Dawley, starting weight approximately 250g), and approximately 1-year-old male rats (Hsd:HanWistar) were used. All animals were under a 12-hour light/dark cycle and had access to *ad libitum* food and water. Mice were housed separately for the duration of the experiment, rats were housed in groups of two. All surgeries and behavioural assays were carried out at the University of Helsinki animal laboratory facilities. All animal experiments were approved by the Finnish National Board of Animal Experiments and were carried out according to the European Community guidelines for the use of experimental animals. License number ESAVI/7812/04.10.07/2015.

4.2.2 PREPARATION, SONICATION, AND INJECTION OF FIBRILS

Mouse PFFs (mPFFs) were removed from -80°C, thawed, and diluted from a 5 mg/ml stock solution using sterile PBS on the morning of the injections. The stock solution of mPFFs were prepared at the lab of Kelvin C. Luk (University of Pennsylvania) as previously (Volpicelli-Daley *et al.*, 2014). Briefly, monomeric mouse α-syn is purified from *E. coli* and then put into a shaker for seven days at +37°C so that they form a fibrillar structure.
For mouse injections, a concentration of 2.5 mg/ml was used and for rat injections 2 mg/ml. The mPFFs were then sonicated (Qsonica Microson XL 2000 Ultrasonic Liquid Processor, Newtown, CT) at power level 2 with a total of 60 pulses, pausing briefly after every 10th pulse. Though a probe tip sonicator was used for the experiments here, we also tested using a sonication bath, with the protocol gained in the lab of Kelvin C. Luk. Using a Bioruptor® water bath sonicator (Diagenode, Belgium) we first vortexed the tube containing the diluted fibrils, then sonicated them on High mode for 10 cycles with 30 seconds on and 30 seconds off. The tubes were gently agitated prior to each injection and kept at room temperature throughout. We checked fibril

size using a Dynamic Light Scattering Zetasizer (Malvern Panalytical, United Kingdom) and electron microscope. It has been shown that fibril length and concentration can affect the outcome of the experiment and that shorter fibrils may be more effective at seeding inclusions and causing dopaminergic system degeneration (Abdelmotilib *et al.*, 2017). We observed that our sonicated fibrils, both with a probe tip and with the sonication bath, were a similar length as reported in the literature (Figure 4, unpublished results, Piotr Chmielarz).

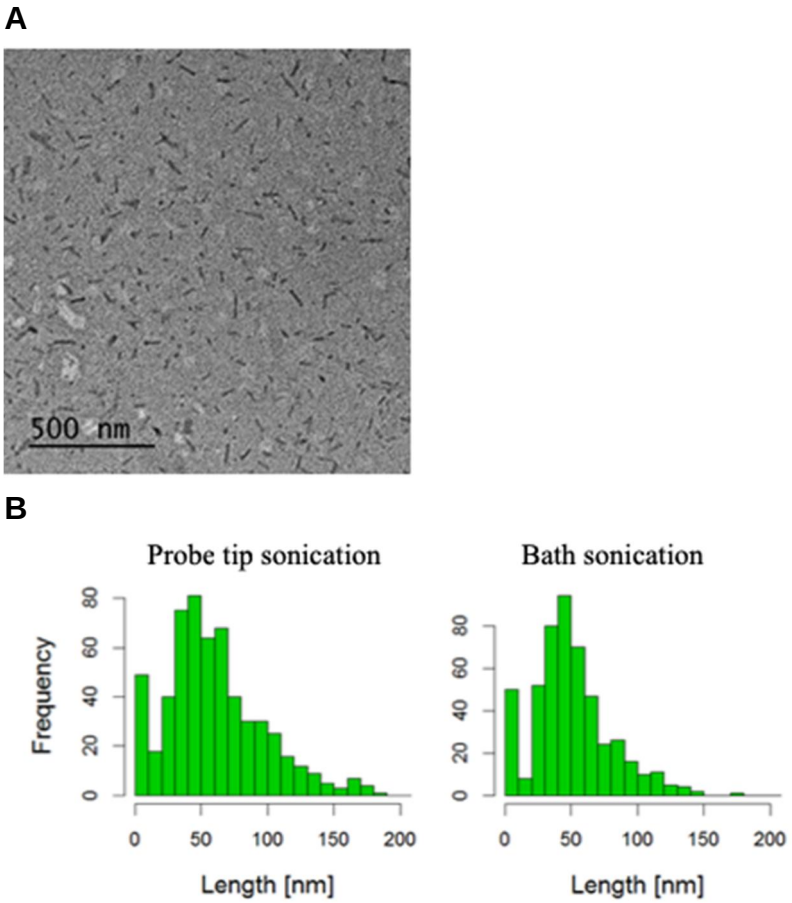


Figure 4. Electron microscopy image (A) and measured length of fibrils (B) after using a probe tip or a bath sonicator. Size distribution is similar between both methods and in accordance with the literature (Piotr Chmielarz, unpublished results).

Animals were anesthetized and placed into a stereotaxic frame for intracranial injections. A 33G steel needle with a 10 µl syringe (Nanofil, World Precision Instruments) was used to inject the fibrils in all experiments. For mice, 2.5 µl was injected into the striatum at a 10° angle (coordinates from bregma: A/P 0.7, M/L -2.2, D/V -3.0 from the skull), with a flow rate of 0.1 µl/minute and letting the needle rest 5 minutes after the injection finished. For rats, 2 µl was injected into 2 sites (total volume 4 µl) of the striatum at a 10° angle (coordinates from bregma: A/P +1.6, M/L -2.8, D/V -6.2; A/P 0.0, M/L -4.1, D/V –6.2 from the skull), using a flow rate of 0.5 µl/minute and letting the needle rest for 5 minutes after each injection. After

the surgery, all animals received carprofen for pain relief (Rimadyl, Pfizer, s.c. 5 mg/kg).

4.2.3 COUNTING OF PS129-POSITIVE ALPHA-SYNUCLEIN INCLUSIONS

At the conclusion of the experiments, brains were removed and cut. The sections were immunostained for an antibody recognizing pS129 α-syn (rabbit anti-pS129 α-syn, Abcam, ab51523, concentration 1:10000). Number of pS129-positive inclusions in the substantia nigra were counted manually by an experimenter trained to recognize pS129-positive staining. 5 to 19 sections from each brain were used from approximately A/P -4.5 to 6.0 relative to bregma. Slides were scanned with Pannoramic 250 Flash II scanner (3DHISTECH, Budapest, Hungary) at the service provided in the Institute of Biotechnology, University of Helsinki. CaseViewer (3DHISTECH) software was used to analyse the sections. The slides were scanned with single layer 20x magnification and the software allows the viewer to easily zoom in and out of whole slide images from 0.1x up to 20x magnification, therefore the pS129-positive staining can be identified easily by eye. Every positive inclusion was then manually marked using the annotation function on each section and the counts are represented as the average number of pS129 inclusions per section. Experimenter was the same for all analyses and was blind to treatments. Brains with absolutely no visible pS129 staining present were removed from the analysis since this indicates an unsuccessful injection of PFFs.

5 RESULTS

5.1 EFFECTS OF AAV-ALPHA-SYNUCLEIN INJECTED ABOVE THE SUBSTANTIA NIGRA OF RATS (II)

We successfully recapitulated the AAV-α-syn model in rats by unilateral injection of AAV carrying human wild-type α-syn above the substantia nigra. This resulted in robust α-syn expression in the midbrain and striatum (publication II, Figure 1c). Similarly to what has been published in the literature (Decressac *et al.*, 2012b; Febbraro *et al.*, 2013), we were able to repeat the striatal TH fibre loss observed, approximately 60%, and the behavioural deficits on the cylinder test, where the rats were using the ipsilateral paw approximately 70% of the total paw use (publication II, Figure 2a, e). However, there was large variation between animals in the degree of loss, as well as in TH+ cells in the substantia nigra pars compacta (publication II, Figure 2d). The striatal loss also showed no significant correlation with amount of α-syn present (publication II, Figure 2c) or behaviour (publication II, Figure 2f, i). We also measured dopamine content and found that it did not correlate with the cylinder behaviour (publication II, Figure 2h).

5.2 AAV DOWNREGULATES TH IN THE SUBSTANTIA NIGRA PARS COMPACTA AFTER INJECTION ABOVE THE SUBSTANTIA NIGRA (II)

We compared three distinct AAVs in a single experiment, all of which used a 1:1 mixture of two serotypes, AAV2/2 and AAV2/5, in order to take advantage of both of their cell entry properties (Davidson *et al.*, 2000):

- AAV-DIO-mCherry, this vector has a double-floxed inverse open reading frame (DIO ORF) encoding fluorescent protein mCherry. This is a Cre-dependent vector, meaning it will not produce protein unless Cre recombinase is present, and since the rats used here are wild-type, no protein should be produced after transduction.
- AAV-eGFP, this vector produces fluorescent protein GFP when injected.
- AAV-α-syn, vector carrying human wild-type α-syn.

The AAV-DIO-mCherry and AAV-eGFP were used here in order to have two controls for AAV-α-syn – one that does not produce protein to act as a negative control and one that does produce protein in order to control for unspecific effects of protein overexpression. In behavioural tests, the AAV-α-syn-injected rats performed similarly as in the above experiments and there were no differences between the three groups (publication II, Figure 3a-b). The same was true for the optical density measurement of TH fibres in the striatum (publication II, Figure 3c). Though in this case the AAV-eGFP group

showed an approximately 20% decrease and the AAV-DIO-mCherry group showed no clear decrease in TH optical density, but there were no significant differences between the groups. However, when dopamine content was measured, we observed a significant drop in the AAV-eGFP group on the injected side which we did not observe in the AAV-α-syn or AAV-DIO-mCherry groups (publication II, Figure 3d). This resulted in a significant difference between the AAV-α-syn and AAV-eGFP groups in dopamine content. Interestingly, all three groups showed TH+ cell loss in the substantia nigra pars compacta using two different counting methods (publication II, Figure 3f-g). There were no significant differences between the groups. Though there was a clear loss of TH+ cells, this was not accompanied by general cell loss since there was only an approximately 20% drop in number of Nissl+ cells in the substantia nigra pars compacta (publication II, Figure 3i). Therefore, we observed a downregulation of TH in the substantia nigra pars compacta cells that was not accompanied by significant fibre loss in the striatum or severe behavioural deficits.

5.3 INJECTION OF ALPHA-SYNUCLEIN PREFORMED FIBRILS RESULTS IN WIDESPREAD PHOSPHORYLATED ALPHA-SYNUCLEIN IN ANATOMICALLY CONNECTED AREAS (III)

We performed experiments in both 3-month-old and one year old wild-type mice and 1-year-old rats where α-syn mPFFs were injected to the striatum and the brains were removed and processed 5-6 months later for immunohistochemistry. Behaviour was performed throughout the experiments. Experimental timeline is shown in Figure 5. For CDNF injections, in mice CDNF was injected one month after PFF injection (Figure 5A), and in rats it was given via chronic infusion one or two months after PFFs (Figure 5B).

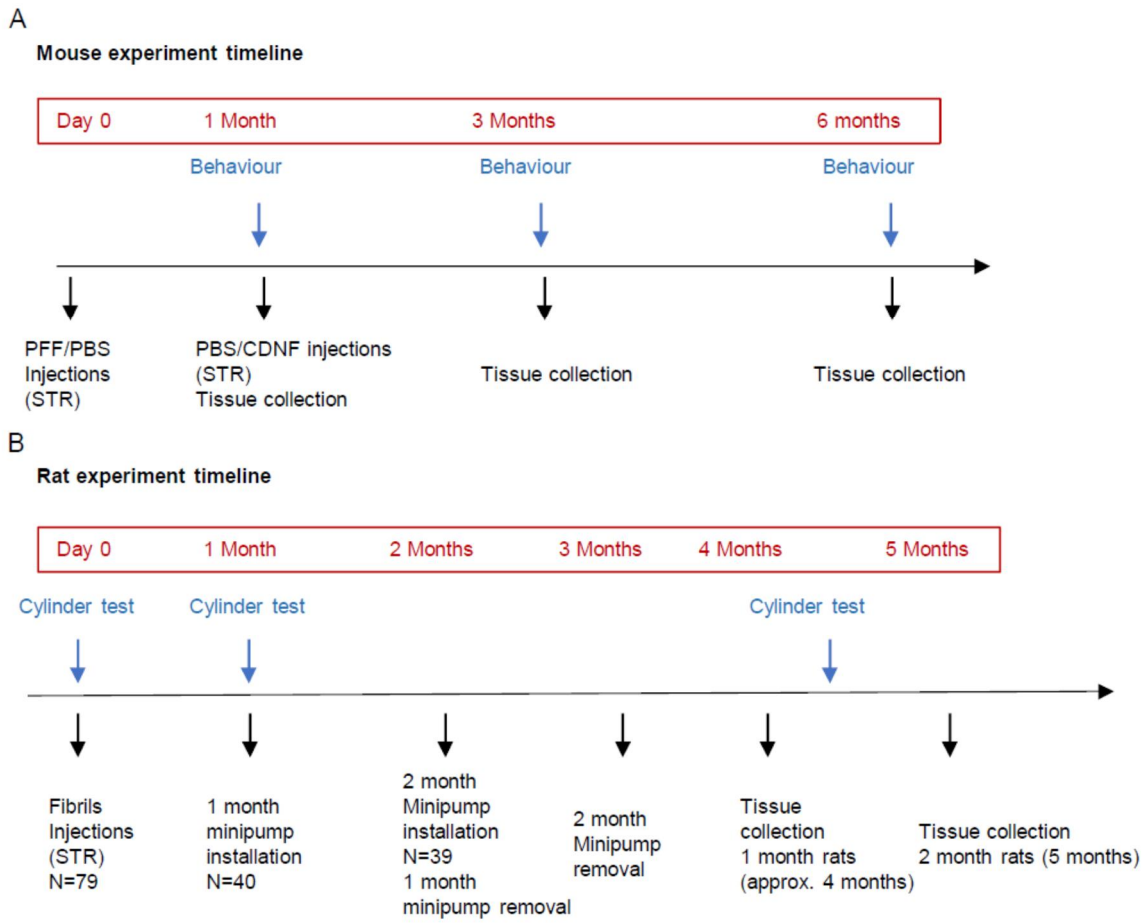


Figure 5. Experimental timelines for α-synuclein preformed fibril experiments. (A) Timeline of experiment for mouse experiments. Preformed fibrils (PFFs) were injected to the striatum (STR) of 3 month and 1-year-old mice at day 0 and 1 month later vehicle (PBS) or CDNF were injected into the same location in the STR. Behavioural tests cylinder test, rotarod, and wire hanger were performed at 1, 3, and 6 months. Animals were euthanized at 6 months and tissue was collected for immunohistochemistry and HPLC. (B) Timeline of experiment for rat experiments. PFFs were injected at day 0 to the STR of 1-year-old rats. Minipumps with vehicle (PBS) or CDNF at two different doses (1.5µg/day or 3µg/day for 30 days) were installed either 1 month after PFF injection or 2 months after PFF injection. After 1 month, the minipumps were removed. Cylinder test was performed at baseline (before fibril injections), at 1 month (before minipump installation), and at 4 months (2 months after minipump installation). Tissue was collected afterwards (4-5 months after fibril injection) for immunohistochemistry and HPLC. Modified from publication III.

In the case of the α-syn PFF model, we obtained prepared mouse PFFs (mPFFs) from the laboratory of Kelvin C. Luk, where they were prepared as previously (Volpicelli-Daley *et al.*, 2014). We diluted, sonicated, and injected them to the striatum of rodents. One to six months after striatal injection, pS129 could be detected in the striatum, cortex, and substantia nigra of the mice (Figure 6A). There was no pS129 immunostaining in mice injected with PBS, nor in α-syn KO mice injected with mPFFs (Figure 6B-C). Therefore, we were able to recapitulate the spreading of the PFFs in this model.

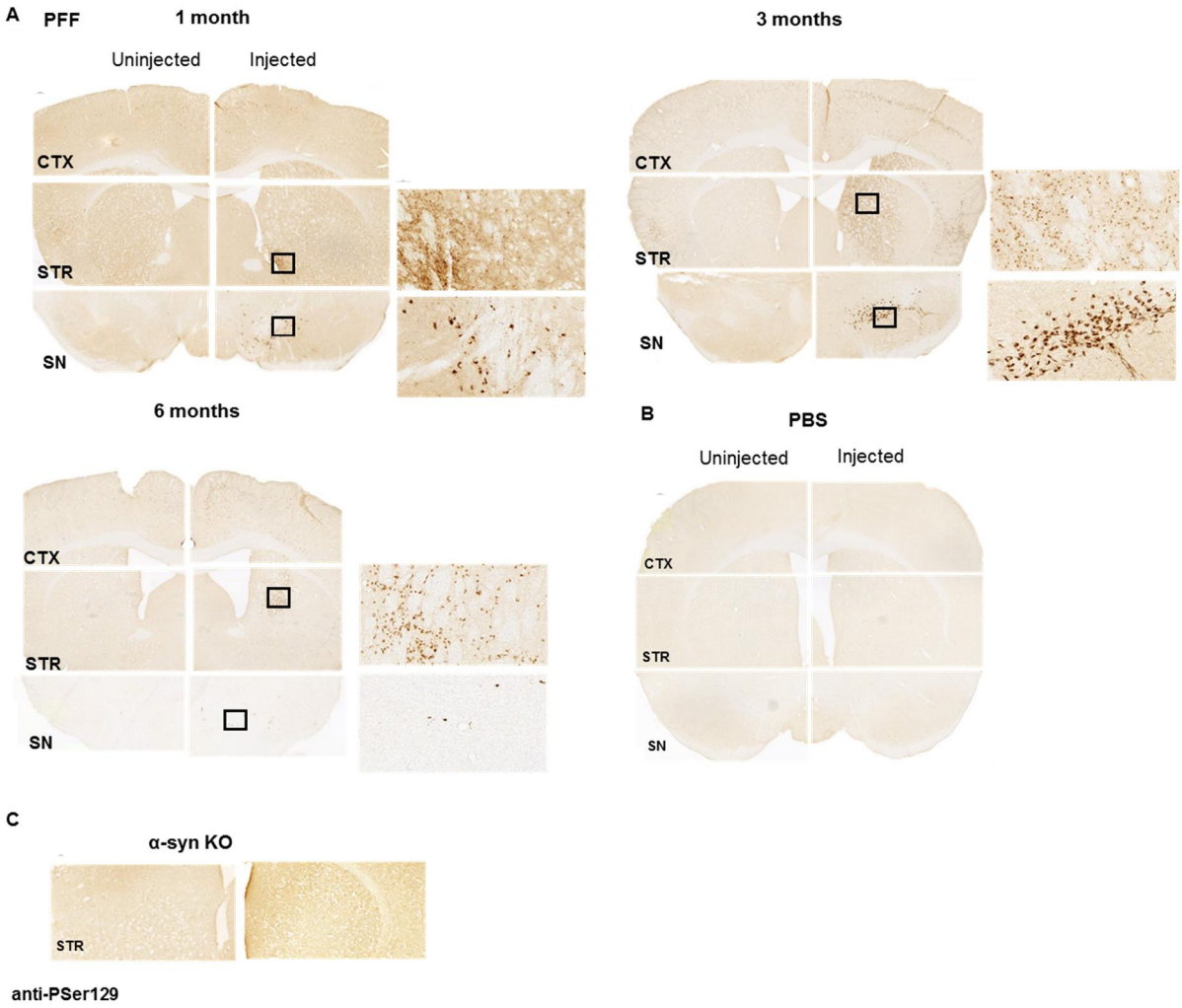


Figure 6. Demonstration of successful fibril induction of phosphorylated α-synuclein and spreading throughout the timepoints. (A) Wild-type mice injected with mPFFs to the striatum and stained for α-synuclein phosphorylated at S129 (pS129) for cortex (CTX), striatum (STR), and substantia nigra (SN) areas at 1, 3, and 6 months after mPFFs. Smaller images 2.5x magnification and close-ups at 40x magnification. (B) Wild-type mouse injected with PBS at 6 months and immunostained with pS129 at 6 months post-injection. No visible staining present, 2.5x magnification. (C) α-synuclein knock-out (KO) mouse injected with mPFFs and immunostained with pS129 at 1 month post-injection. No visible staining present, 10x magnification. Modified from publication III.

5.4 EFFECTS OF ALPHA-SYNUCLEIN PREFORMED FIBRILS AND TREATMENT WITH CDNF IN MICE AND RATS (III)

Mice at approximately three months old and one year old were injected with mPFFs or PBS and behaviour was performed at one, three, and six months post-mPFF injection. At one month after mPFF injection, PBS or CDNF were injected. At one month, adult mice injected with mPFF spend significantly less time on rotarod than naïve mice (Figure 7A, unpaired t-test, t=4.172 df=30, p=0.0002). In the coat hanger test, 3-month-old mice did not show significant differences between PBS and mPFF injections (Figure 7B,

unpaired t-test, t=1.209 df=58, p=0.2315). At 1 month, there is no clear decrease in TH optical density (Figure 7C, average=95.55%, SEM=3.92%). At three months after injections, 3-month-old mice also had a significant difference on rotarod between naïve and mPFF (Figure 7D, unpaired t-test, t=5.199 df=22, p=<0.0001). Additionally, on cylinder test performed at three months for 3 month old mice, there was a significant difference in ipsilateral paw use between the PBS and CDNF groups, where CDNF treated animals were using both paws more evenly compared to PBS treated, in other words PBS mice were using the ipsilateral paw significantly more than the contralateral, and the CDNF mice were not (Figure 7E, two-way ANOVA, column factor F (2, 42) = 21.51, p=<0.0001. Tukey's multiple comparison test as post-hoc, PBS ipsilateral vs. PBS contralateral, p=0.0051; CDNF ipsilateral vs. CDNF contralateral, p=0.7109). There were no significant changes in three months post-mPFF injection on TH optical density (Figure 7F, three months, one-way ANOVA, F (2, 21) = 0.6828, p=0.5161), though this is expected since it has been shown that the mice do not show TH loss until 6 months post-injection (Luk *et al.*, 2012). However, at 6 months there are also no significant differences between the groups on TH+ fibre density (Figure 7G, one-way ANOVA, F (4, 23) = 2.782, p=0.0508). Although both time points show presence of pS129 inclusions (Figure 6).

For one-year-old mice, at one month there is no significant difference on rotarod between PBS-injected mice and mPFF (Figure 8A, unpaired t-test, t=0.7135 df=38, p=0.4799). Also, in the coat hanger test, one-year-old mice did not show significant differences between PBS and mPFF injections (Figure 8B, unpaired t-test, t=1.712 df=38, p=0.095). On wire hanger time at three months for one-year-old mice, two months after PBS/CDNF injections, there was a significant difference between the mice that received PFF and then PBS or CDNF, where the CDNF-treated animals performed significantly better than PBS-treated (Figure 8C, one-way ANOVA, F (3, 36) = 3.046, p=0.0410, Tukey's multiple comparison test, PFF+PBS vs. PFF+CDNF p=0.0405).

We also modeled PFFs in one-year-old rats. PFFs were injected into the striatum and one or two months later CDNF was given via minipump to the striatum at two concentrations: 1.5µg/day for 30 days or 3µg/day for 30 days, with PBS as a vehicle (1xPBS/day for 30 days). The cylinder test was used as an outcome measure. At baseline (before fibril injection) and 1 month (before minipump installation) there were no significant differences between treatments over time for contralateral paw use (Figure 8D, two-way ANOVA, treatment: F (2,58)=2.788, p=0.0698; time: F (2,29)=2.448, p=0.1041; interaction: F (4, 58)=2.048, p=0.0995). At 4 months however (2 months after minipump installation), there was a significant difference between PBS and CDNF 3µg/day on contralateral paw use in post hoc testing (two-way ANOVA, Tukey's post hoc test for multiple comparisons, p=0.0114). At four or five months after mPFF injection, brains were removed and dopamine concentration was measured on HPLC. For the one month minipump experiment, there was no significant difference between the uninjected side and injected side for the PBS (Figure 8E, two-way ANOVA, column factor, F (2, 64) = 0.9194, p=0.4040. Tukey's multiple comparison test as post-hoc, PBS uninjected vs. injected side p=0.8451). There was a significant difference between sides for the CDNF 1.5µg/day treatment (Figure 8E, two-way

ANOVA, row factor, F (1, 64) = 10.61, p=0.0018. Tukey's multiple comparison test as post-hoc, CDNF 1.5µg/day uninjected vs. injected side p=0.0195), and there was no significant difference between the sides for the CDNF 3µg/day (Figure 8E, two-way ANOVA, column factor, F (2, 64) = 0.9194, p=0.4040. Tukey's multiple comparison test as post-hoc, CDNF 3µg/day uninjected vs. injected side p=0.8315). For the counting of pS129+ inclusions four months after mPFF injection, there were no significant differences between treatments (Figure 8F, one-way ANOVA, F (2,31)=0.6597, p=0.5241).

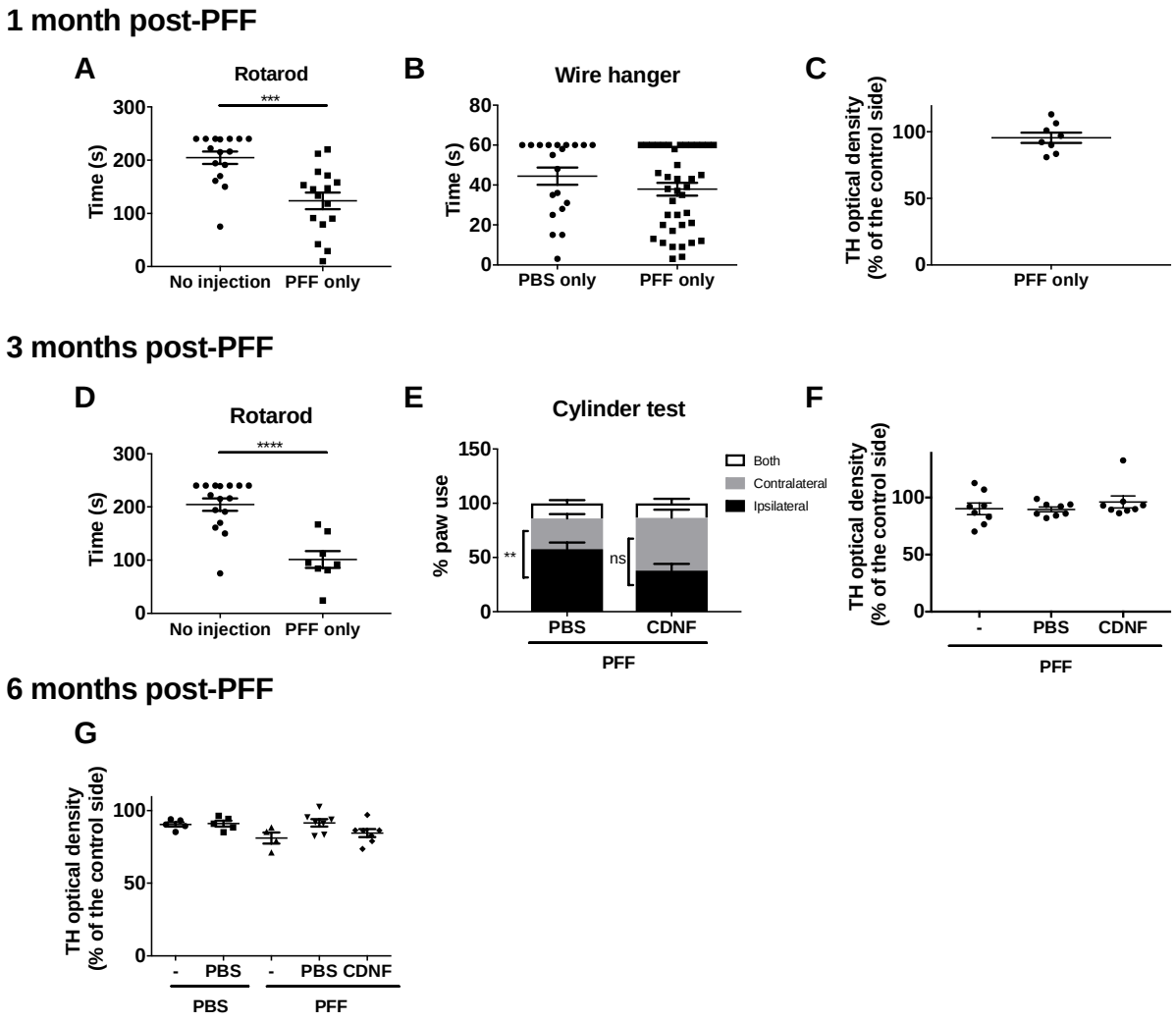


Figure 7. Behaviour and pathology outcome after CDNF treatment in fibrils injected mice. (A-G) Mouse behaviour and tyrosine hydroxylase optical density for 3-month-old mice at time of injection. (A) Rotarod at 1 month after injection. No injection indicates naïve mice, PFF only denotes fibrils were given at day 0. (B) Wire hanger time at 1 month after injection. PBS only and PFF only indicate that the injections were given on day 0. (C) Tyrosine hydroxylase optical density in the striatum as a percent of the control side at 1 month after PFF injection. (D) Rotarod at 3 months after injection. No injection indicates naïve mice, PFF only denotes fibrils were given at day 0. (E) Cylinder test at 3 months after injection, graph represents ipsilateral, contralateral, as well as both paw touches on the cylinder wall. PFF+PBS/PFF+CDNF indicates that PFFs were given at day 0 and PBS/CDNF was given 1 month later. (F) Tyrosine hydroxylase optical density in the striatum as a percent of the control side 3 months after fibrils. PFF only indicates PFFs given at day 0, and PFF+PBS/CDNF fibrils given at day 0 and PBS/CDNF 1 month later. (G) Tyrosine hydroxylase optical density in the striatum as a percent of the control side. PBS/PFF only indicates PBS or PFFs given at day 0 and no other injections, PBS+PBS indicates PBS given

at day 0 and again 1 month later, PFF+PBS/CDNF indicates fibrils at day 0 and PBS/CDNF 1 month later. Bars represent mean ± SEM. Figure modified from publication III.

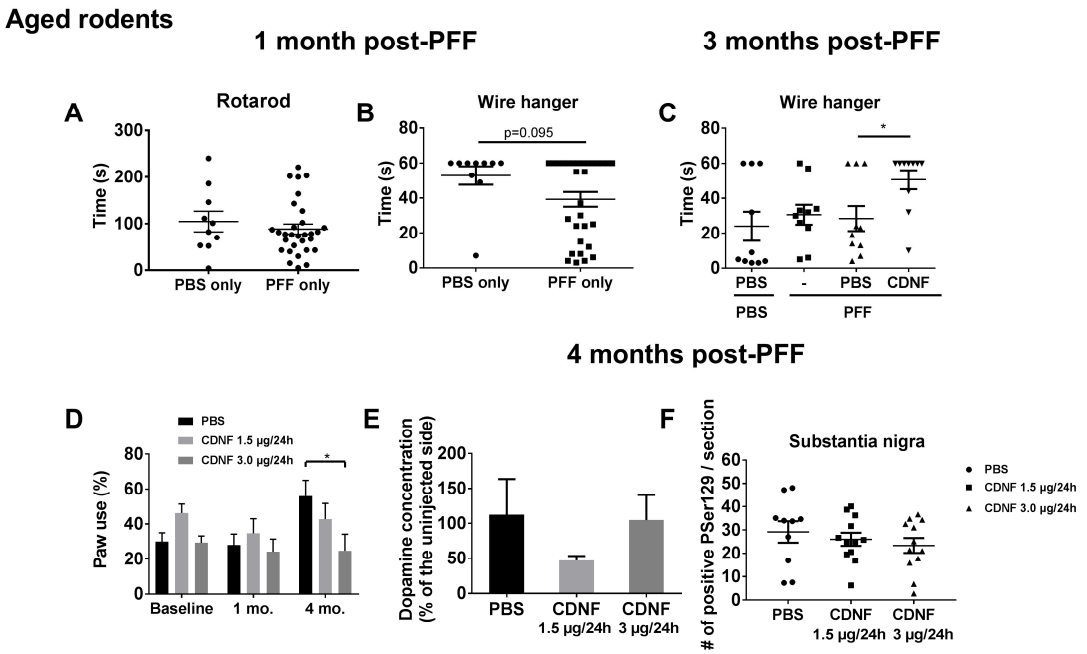


Figure 8. Behaviour and pathology outcome after CDNF treatment in fibrils injected mice and rats, rodents one year old at time of injection. (A-C) Mouse behaviour for 1-year-old mice at time of injection. (A) Rotarod 1 month after injection. PBS/PFF only indicates PBS for fibrils given at day 0. (B) Wire hanger time 1 month after injection. PBS/PFF only indicates PBS or fibrils given at day 0. (C) Wire hanger time 3 months after injection. PBS/PFF only indicates PBS or PFFs given at day 0 and no other injections, PBS+PBS indicates PBS given at day 0 and again 1 month later, PFF+PBS/CDNF indicates fibrils at day 0 and PBS/CDNF 1 month later. (D-F) Behaviour and pathological outcome for 1-year-old rats at time of injection. (D) Contralateral paw use for rats (percent of total paw use). Baseline indicates before fibrils were injected, 1 month is 1 month after fibrils but before installation of minipumps, and 4 months is 4 months after fibrils and 3 months after treatment. Minipumps administer treatment to the striatum for 30 days. (E) Dopamine concentration in the striatum as a percentage of the control side at 4 months after fibril injection. (F) Number of pS129 inclusions in the substantia nigra for each treatment 4 months after fibril injections. Data is presented as the average number of inclusions per section counted. Bars represent mean ± SEM. Figure modified from publication III.

5.5 EFFECTS OF A SINGLE INJECTION OF CDNF TO THE SUBSTANTIA NIGRA OR IN COMBINATION TO THE STRIATUM IN THE 6-OHDA MODEL IN RATS

There were no significant weight changes after injections of human CDNF and 6-OHDA to rats throughout the experiment (Figure 9A). The amphetamine-induced rotations over the time-course of the experiment are shown in Figure 9B. At 8 weeks post-lesion, both CDNF treatments showed a significant decrease in amphetamine-induced rotations compared to vehicle (Two-way ANOVA, treatment effect, $F(2, 50) = 6.207$, $p=0.0039$, time effect, $F(2, 100) = 4.929$, $p=0.0091$, treatment X time interaction, $F(4, 100) = 1.85$, $p=0.1251$; post-hoc Tukey's multiple comparison test, CDNF 3µg SN $p=0.007$, CDNF combination STR+SN $p=0.0003$) (Figure 9B). However, 12 weeks post-lesion, only the combination of striatal and nigral CDNF

treatment showed a significant difference compared to vehicle (Two-way ANOVA, treatment effect of, F (2, 50)=6.207, p=0.0039; post-hoc Tukey's multiple comparison test, CDNF 3µg SN p=0.1102, CDNF combination p=0.0254) (Figure 9B). Total ipsilateral paw use at each measured time point is presented in Figure 9C. At 4 weeks post-lesion, rats injected with 3 µg of CDNF to the substantia used the ipsilateral paw significantly less than vehicle treated rats (One-way ANOVA, F (2, 40) = 4.065, p= 0.0461, Dunnett's multiple comparison as post-hoc test). There were no significant differences in ipsilateral paw use between either of the CDNF treated groups at 8 weeks (One-way ANOVA F (2, 41) = 0.2777, p= 0.7590) or 12 weeks (One-way ANOVA, F (2, 39) = 0.3026, p= 0.7406) post-lesion compared to vehicle.

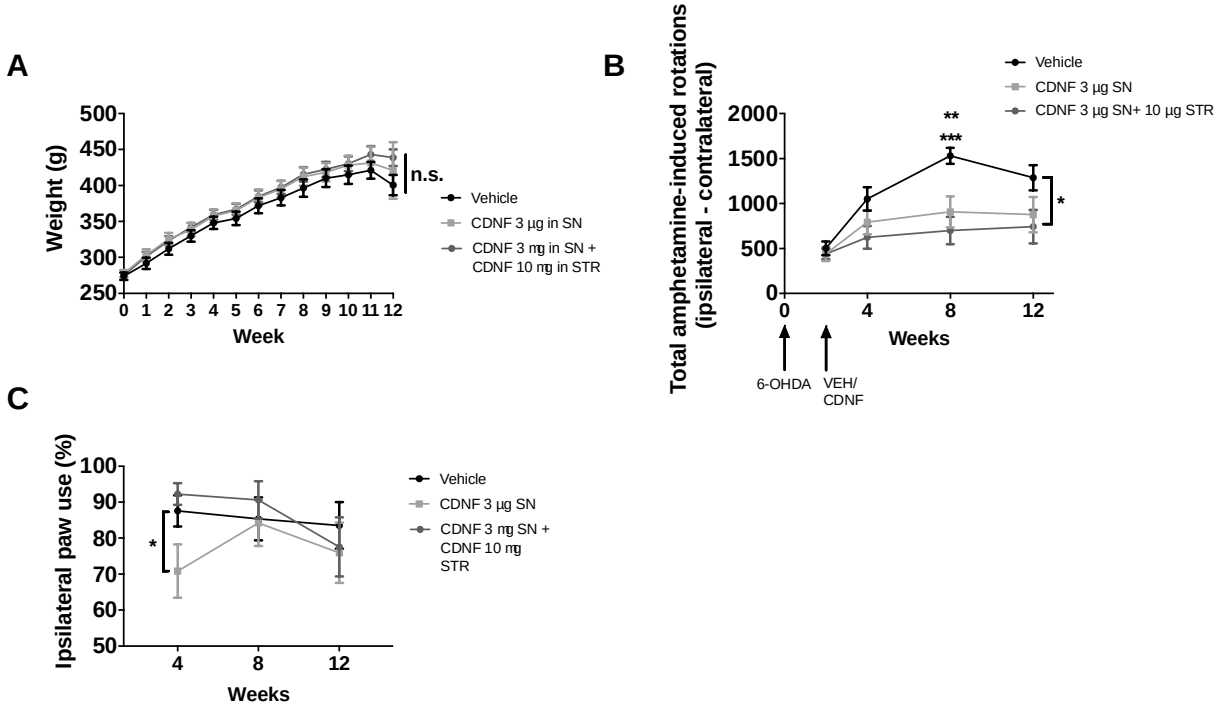


Figure 9. Amphetamine-induced rotations and cylinder test after 6-OHDA and CDNF administration. (A) Weight of animals throughout the entire experiment. No significant differences at any of the time points between treatment groups were observed. (B) Time course of rotations from 2 to 12 weeks. 6-OHDA was given at week 0 and 2 weeks later the CDNF treatments were administered. At 8 weeks after 6-OHDA, there was a significant effect of both treatments (Two-way ANOVA, post-hoc Tukey's multiple comparison test,*CDNF STR 10µg+SN 3µg, p=0.0254; **CDNF SN 3µg, p=0.007; ***CDNF STR 10µg+SN 3µg, p=0.0003). Vehicle n= 22; CDNF SN 3µg n=16; CDNF STR 10µg+SN 3µg n=15. (C) Time course of cylinder test from 4 to 12 weeks representing the total ipsilateral paw use (ipsilateral touches / ipsilateral + contralateral + both touches). There was a significant difference for rats treated with 3µg of CDNF to SN at 4 weeks (One-way ANOVA, post-hoc Dunnett's multiple comparison, *CDNF SN 3µg, p= 0.0461). Vehicle n= 19; CDNF 10µg STR n=15; CDNF SN 3µg n=13; CDNF STR 10µg+SN 3µg n=11. Bars represent mean ± SEM. Figure from unpublished results.

A severe lesion model was used here (Back *et al.*, 2013a; Penttinen *et al.*, 2016), where CDNF as a single injection has not been tested before. Consequently, no significant differences were seen between vehicle and CDNF treatments in the optical density of TH immunoreactive fibres in the striatum at 12 weeks post-lesion (One-way ANOVA, F (2, 50) = 1.039, p= 0.3613) (Figure 10B) or in the number of TH+ cells in the substantia nigra

pars compacta (One-way ANOVA, F (2, 49) = 1.42, p= 0.2515) (Figure 9D) 12 weeks after the lesion. Additionally, DAT immunoreactive striatal fibres were analysed (Figure 10E), however, no significant differences between vehicle and CDNF treatments were observed at 12 weeks post-lesion (One-way ANOVA, F (2, 32) = 0.9134, p=0.4114) (Figure 10F).

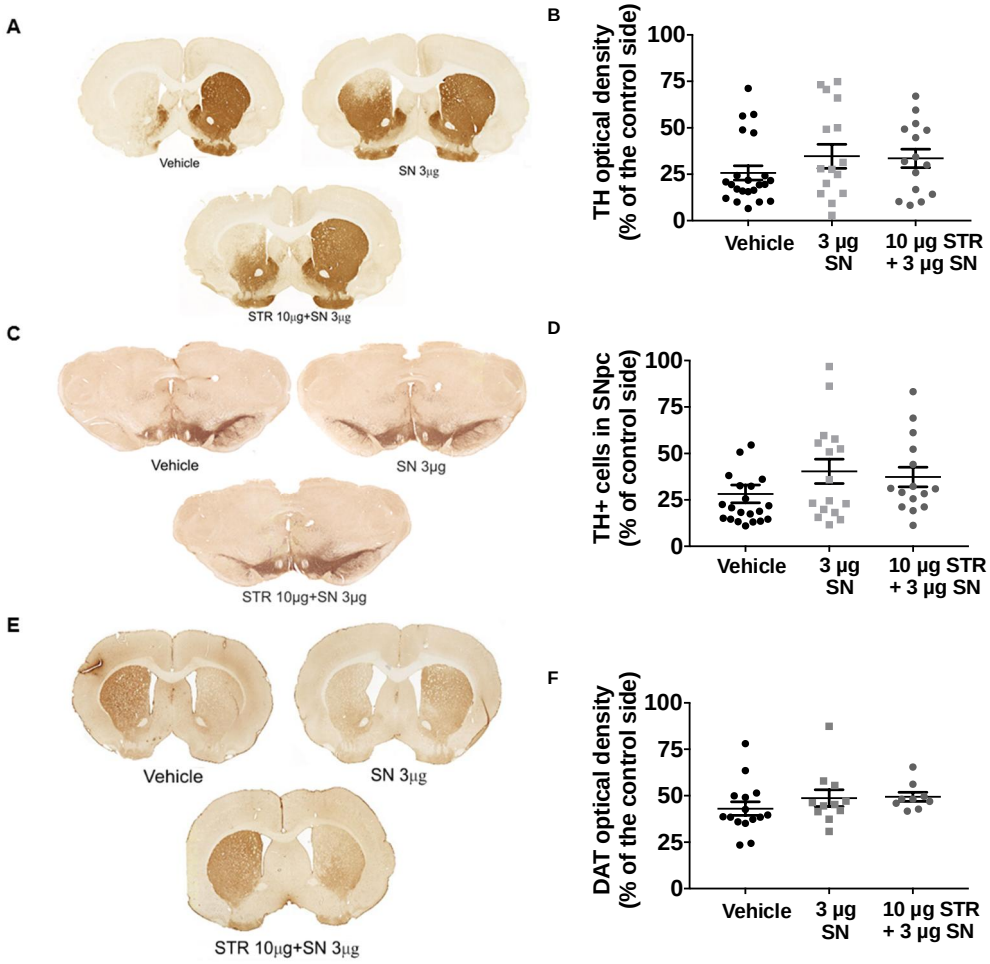


Figure 10. Dopamine system outcome measures. Amount in µg indicates amount of CDNF protein. (A) Representative striatal slices with TH immunohistochemistry for each treatment group, magnification 1x. (B) TH optical density of the striatum represented as a percent of the control (non-injected) side. (C) Representative nigral slices with TH immunohistochemistry for each treatment group, magnification 1,5x. (D) The number of TH-positive cells in the substantia nigra as a percent of the control (non-injected) side. (E) Representative striatal slices of DAT immunohistochemistry for each treatment, magnification 1x. (F) DAT optical density of striatum as a percent of the control (non-injected) side. Values are represented as a percent of the non-injected side as they are not absolute cell counts. Bars represent mean ± SEM, statistical analysis using one-way ANOVA. Figure from unpublished results.

5.6 DIFFUSION AND LOCALIZATION OF CDNF AFTER INJECTION TO THE RAT SUBSTANTIA NIGRA (IV)

To quantitatively study the diffusion and transport of intrastriatally injected CDNF to other brain areas, and to repeat the previously published data (Voutilainen *et al.*, 2011), as well as have a positive control, ^{125}I-labelled

CDNF was injected to the striatum of rats, and 24 hours later dissected brain areas were measured in a gamma counter (Figure 11A-B). CDNF spread intensely to the frontal cortex of the injected side, while there was little spread of ^{125}I-CDNF to other cortical areas. CDNF also spread well to the ipsilateral and contralateral hippocampus, and substantia nigra (Figure 11A-B). Next, we wanted to analyze CDNF diffusion after nigral administration and possible anterograde transport from substantia nigra to striatum which has not been studied previously. CDNF labelled with ^{125}I was injected into the substantia nigra of rats and brain areas dissected 24 hours later were measured in a gamma counter (Figure 11C-D). CDNF diffused most strongly to the ipsilateral STN from the substantia nigra. The radioactivity in the ipsilateral substantia nigra was the highest overall, with it being significantly different than the other brain areas (excluding the STN) (One-way ANOVA, $F(11, 52) = 8.6$, $p = <0.0001$) (Figure 11C). To study whether the spread of CDNF to the STN was due to passive diffusion or active transport, ^{125}I-CDNF was injected to the substantia nigra together with two doses of unlabelled CDNF (2000-fold molar excess or 10000-fold molar excess) to compete for binding and anterograde axonal transport of the labelled CDNF. The diffusion/transport of ^{125}I-CDNF from the substantia nigra to the STN was not blocked by either of the doses of unlabelled CDNF (One-way ANOVA, $F(2, 18) = 0.0068$, low: $p=0.9801$, $df=52$; high: $p=0.9928$, $df=52$) (Figure 11E) which suggests passive diffusion of CDNF after nigral administration.

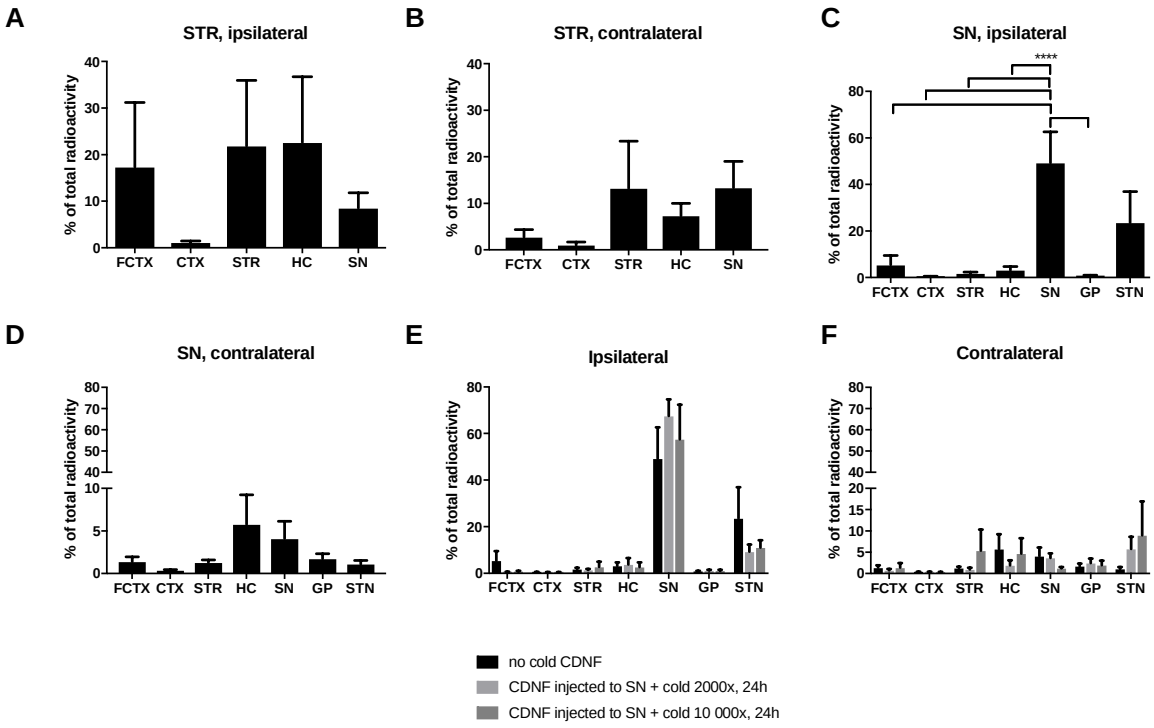
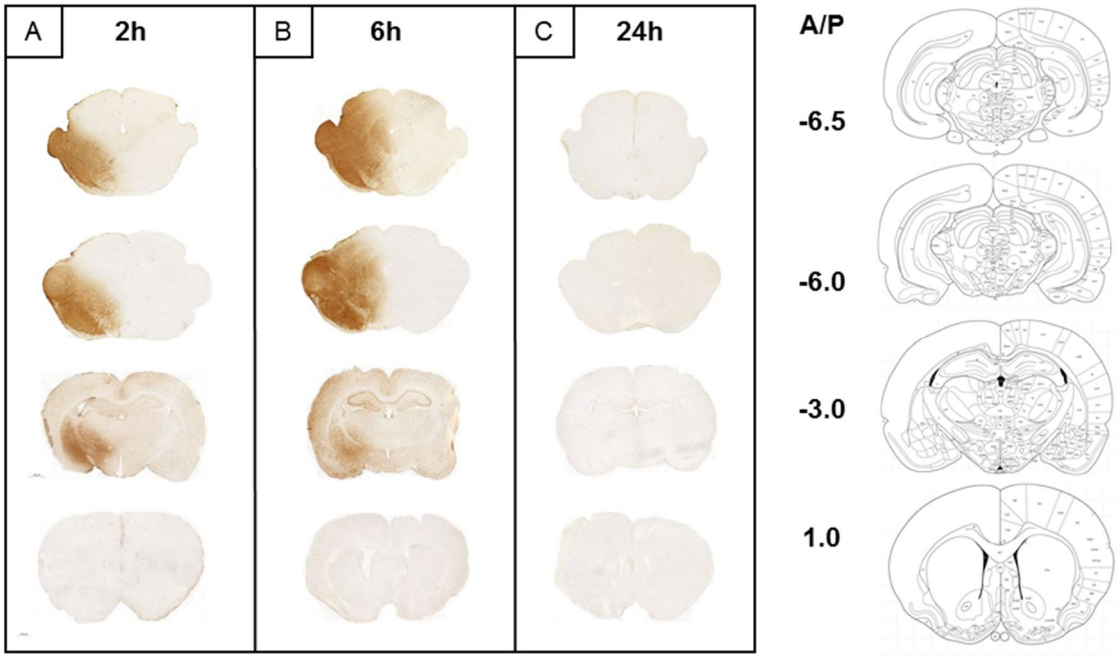


Figure 11. Diffusion of ^{125}I-CDNF when injected to the striatum or substantia nigra of naïve rats**.** A-B) Percentage of the total radioactivity (calculated based on radioactivity of all brain areas measured) after injecting radiolabeled CDNF into the STR of naive rats and measuring 24 h later, ipsilateral regions (A) and contralateral (B). C-D) Percentage of the total radioactivity (calculated based on radioactivity of all brain areas measured) after injecting radiolabeled CDNF into the SN of naive rats and measuring 24 h later, ipsilateral regions (C) and contralateral (D). E-F) 2000x or 10 000x molar excess of cold (unlabeled) CDNF was given together with radiolabeled CDNF to SN of naïve rats and measured 24h later. Ipsilateral regions (E) and contralateral (F). *(n*=5-6/brain

region) Bars represent mean ± SEM. ****p=<0.0001. FCTX= frontal cortex, CTX= cortex, STR= striatum, HC= hippocampus, SN= substantia nigra, GP= globus pallidus, STN= subthalamic nucleus. Figure modified from publication IV.

When CDNF protein was injected to the substantia nigra of the naïve rat brain and monitored at different time points in order to characterize the diffusion after nigral injection, we observed robust CDNF staining in the ipsilateral midbrain, including the substantia nigra, at the 2 (Figure 12A) and 6 (Figure 12B) hour time points, but not at the 24 hour time point (Figure 12C). Additionally, CDNF protein appeared to diffuse anteriorly and posteriorly from the substantia nigra at 2 and 6 hours, but again was not detected at 24 hours. At the 2- and 6-hour time points, there was clear staining in the hippocampus and amygdala, as well as thalamic and hypothalamic regions. There was no staining observed in the dorsal striatum at any of the time points. Additionally, increasing the volume of the CDNF injection while keeping the total amount of protein constant, increased its diffusion in the substantia nigra. When 1 or 4 µl of CDNF was injected to the substantia nigra of naïve rats, the higher volume appeared to diffuse farther after 2 hours as compared to the lower volume (Figure 12D). It can also be observed that at higher magnifications there are concentrated, darkly stained, puncta throughout the injected area (Figure 12E, black arrow heads).



hCDNF immunostaining

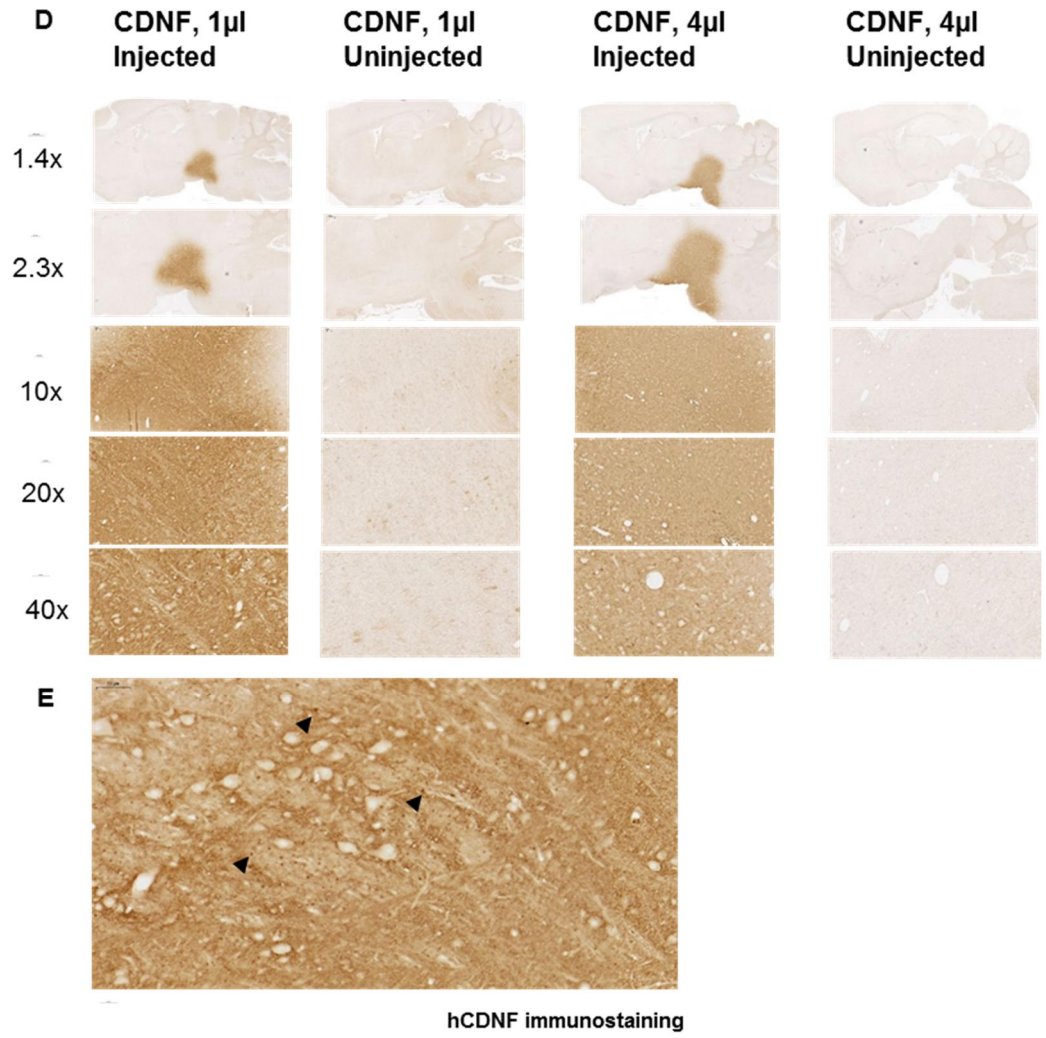
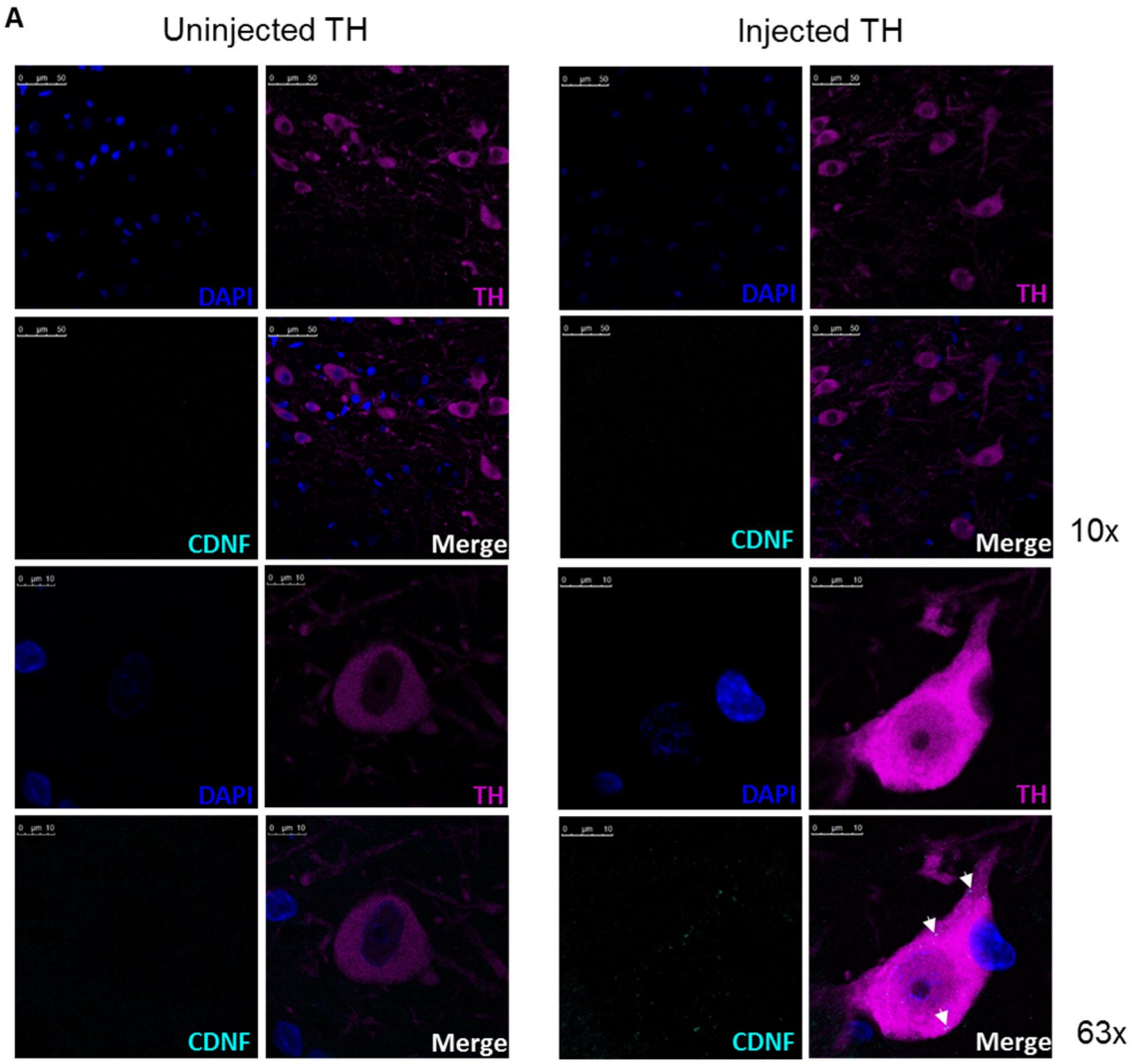


Figure 12. Diffusion of human CDNF after injection to the substantia nigra in naïve rat brain. CDNF (3µg in 4µl) was injected to the SN of rats and perfused (A) 2, (B) 6, or (C) 24 hours later. Human CDNF (hCDNF) immunostaining of a representative posterior, nigral, thalamic, and striatal section for each time point. N=2/time point. (D) Diffusion of human CDNF (3µg) when injected into the SN in the volume of 1µl or 4µl. Rats were perfused 2 hours after the injection. Images shown at increasing magnification of the CDNF injected side versus the uninjected side. (E) 40x magnification of injected area. Black arrowheads indicate dark brown puncta staining of CDNF. Figure modified from publication IV.

Next, we wanted to determine whether CDNF colocalized with TH+ neurons or parvalbumin (PV) neurons using immunofluorescent staining and confocal microscopy. Brains from six rats were analyzed to clarify whether CDNF colocalized with either of the neuronal subtypes. Out of several cells detected in the nigral area of each brain, it can be observed that there are CDNF immunoreactive puncta (cyan, white arrows) inside the TH+ neurons (magenta, Figure 13A) on the injected side. By contrast, from the PV stained sections, CDNF is diffused around the neurons but does not appear to colocalize with them as clearly as in the TH (Figure 13B). Additionally, as can be observed in Figure 12, there is diffuse CDNF staining all around the midbrain, including the substantia nigra. The confocal images show the same:

CDNF is diffused around the cells and only some cells hold CDNF-immunoreactive puncta as demonstrated in Figure 12.



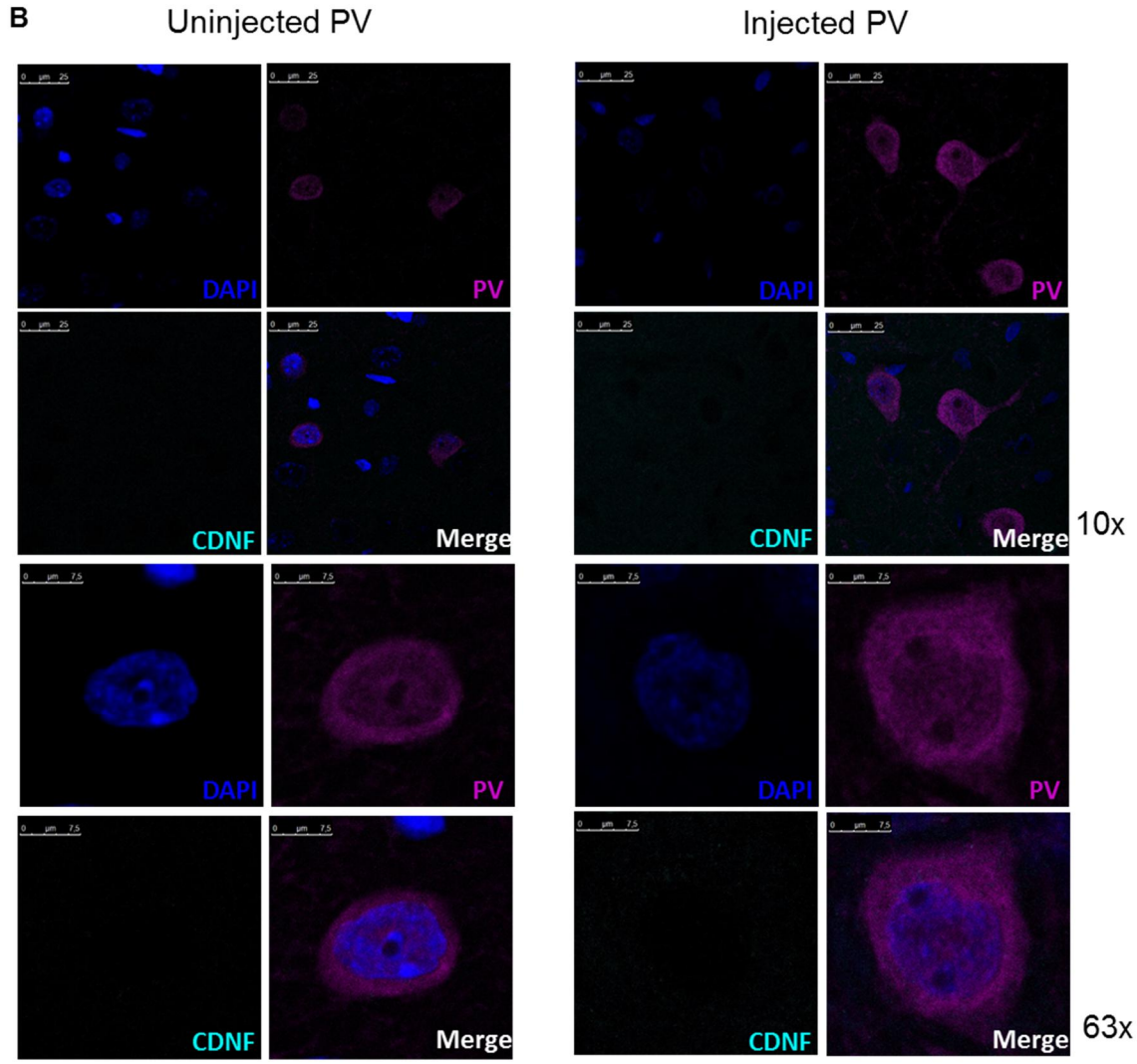


Figure 13. Representative confocal images of CDNF-injected rat substantia nigra with TH neurons or PV neurons to demonstrate colocalization. **(**A) Representative confocal images from a rat brain injected with human CDNF into the substantia nigra and perfused 2 hours later with costaining for CDNF and TH. Total of 6 rats were injected and stained for the colocalization study. White arrows in the injected side indicate CDNF immunoreactive puncta (cyan dots). Uninjected side representative image taken with identical settings to account for possible background. Top panel 10x objective, lower panel 63x objective. (B) Representative confocal images from rats injected with human CDNF into the substantia nigra and perfused 2 hrs later with costaining for CDNF and PV (parvalbumin). Uninjected side representative image taken with identical settings to account for possible background. Top panels 10x objective, lower panels 63x objective, N=6. Figure modified from publication IV.

5.7 BEHAVIOUR AND HISTOLOGICAL OUTCOMES OF LACTACYSTIN-INJECTED MICE (V)

In addition to the three models used above, we used a lactacystin model to induce dopamine neuron degeneration and buildup of α-syn. We were able to recapitulate the model in approximately 2-month-old and 1-year-old mice by injecting lactacystin to the right substantia nigra (publication V). Specifically, the mice showed increased use of the ipsilateral paw on the cylinder test (publication V, Figure 1a-b). They also had a unilateral loss of

TH in the striatum and substantia nigra, as well as decreased dopamine and DOPAC (3,4-dihydroxyphenylacetic acid) content in the striatum (publication V, Figure 2). The mice also showed increased neuroinflammatory markers GFAP (glial fibrillary acidic protein) and Iba1 (ionized calcium binding adaptor molecule 1), markers for astrocytes and microglia, respectively, that were spread throughout the ipsilateral side (publication V, Figure 3), and increased α-syn immunoreactivity in the injected substantia nigra (publication V, Figure 5). However, whether these forms were insoluble could not be verified in this study.

6 DISCUSSION

We set out to utilize and validate α-syn-based Parkinson's disease models to test CDNF and other therapies. Additionally, we performed studies to complement previous CDNF characterization studies. While we were able to repeat what has been published for these models, we have also outlined some drawbacks to them; and in regard to CDNF therapy, we have shown some positive effects in both toxin and α-syn-based models.

6.1 AAV-ALPHA-SYNUCLEIN MODEL (II)

The AAV-α-syn model has been extensively utilized in rodents (see Table 3) to unilaterally overexpress human wild-type α-syn in the nigrostriatal tract and incur TH loss as well as typical behavioural deficits used in Parkinson's disease toxin models such as cylinder test and rotational assays. It has been touted as a more physiologically relevant model for Parkinson's disease research than toxin models since there is α-syn present. In our studies, we were able to repeat across several experiments the TH loss and behavioural deficits on the cylinder test that have been published previously (publication II). However, the high variation between animals and lack of correlation between outcome measures would make it a difficult model to test therapies on. Since the degree of TH loss in the AAV-α-syn-injected animals did not correlate with behaviour on the cylinder test or amount of α-syn present, from these alone it would be impossible to determine which animals had a successful lesion. Therefore, other behaviour tests would need to be tested to determine if they had a higher correlation with TH loss. Or then a method to test in live animals whether there is a deficit in the dopamine system would be useful, such as SPECT/CT technology which combines single-photon emission computed tomography and computed tomography, and radioligands for DAT (Back *et al.*, 2013b). Although this would not solve the issues we encountered when overexpressing control AAVs.

Toxicity to cells with overexpression of high levels of AAV-eGFP has been reported previously (Klein *et al.*, 2006), as well as a robust immune response (Samaranch *et al.*, 2014). In the latter study, AAV9-eGFP from jellyfish injected into non-human primates resulted in cell death and an increase in reactive astrocytes and microglia at the transduced area in the striatum. Also, the two animals that received AAV9-eGFP to the cisterna magna had decreased appetite, ataxia, and impaired balance and hand-eye coordination, which was most likely a result of deficits in cell function in the Purkinje cells in the cerebellum as well as astrocyte and microglia activation there. By contrast, the non-human primates that received AAV9-AADC from human showed no such issues and appeared similarly to the control animal in behaviour and histology. This is unsurprisingly since GFP is a non-mammalian protein, and though it is widely used as a control in biological studies, its incompatibility with non-human primates and rodents is clear. Related to this, AAV-eGFP is often used as a control in AAV-α-syn studies, and it has been shown that if it is expressed at similar levels to AAV-α-syn it

will cause loss of TH+ substantia nigra pars compacta cells (Koprich *et al.*, 2011; Landeck *et al.*, 2016). In fact, we observed this in publication II, where AAV5-eGFP caused a higher degree of TH loss in the striatum and resulted in a high number of amphetamine-induced rotations compared to AAV5-α-syn. When we explored this further, we found that when we compared AAV2/2 and AAV2/5 mixtures of AAV-α-syn, AAV-eGFP, and AAV-DIO-mCherry, where the latter should not express protein in wild-type animals since they lack Cre recombinase, that all three vectors downregulated TH in the substantia nigra pars compacta neurons on the injected side. While this result was clear from visual examination of the sections, we quantified it using two validated methods for counting of TH+ cells in the substantia nigra pars compacta (Penttinen *et al.*, 2016; Penttinen *et al.*, 2018a). We also quantified the Nissl+ cells in the substantia nigra pars compacta and found only a small decrease, indicating that this seems to be a loss of the TH phenotype rather than a global loss of cells. This loss of phenotype has been observed with long term overexpression of GDNF via AAV (Georgievska *et al.*, 2002; Penttinen *et al.*, 2018b). We can hypothesize how it may be occurring here, one possibility is that since TH is a highly regulated enzyme in dopamine synthesis it is easily downregulated when AAV is applied. If this is the case, a different marker of dopamine would be needed here to more accurately measure the degree of loss, such as VMAT2 which has been used rarely in AAV-α-syn studies as a secondary marker to TH (Decressac *et al.*, 2011). However, in its current form the AAV-α-syn model is not an ideal model for an α-syn-based Parkinson's disease animal model where issues with between animal variation, controls, and its dissimilarity to the human disease, need to be considered. Since α-syn is expressed here at very high, non-physiological levels, we can conclude that it is causing downregulation and dysfunction to a degree that would not be observed in disease. While it is of course common to use models that show faster disease onset for practical purposes, there is likely a more useful model than AAV overexpression that would not cause toxicity to cells due to high protein levels. However, if one would want to use this model, a better control is needed. We have recommended proteins that are derived from mammals that would form oligomers that are not prone to misfolding, such as cytoskeletal proteins actin or collagen.

6.2 PREFORMED ALPHA-SYNUCLEIN FIBRILS MODEL AND CDNF (III)

The PFF model of α-syn-based Parkinson's disease in rodents has been characterized only relatively recently as of this writing, but it is increasingly being used by researchers. When the PFFs are injected into the animal it has a slower onset than toxin or AAV models, results in dopamine loss and motor deficits, and it often shows α-syn spreading from its injection location. Here, we were able to reliably show the spread of pS129 α-syn in the mouse and rat brain, as well as some motor deficits (publication III). However, loss of TH was relatively absent in mice (publication III). This was in contrast to (Luk *et al.*, 2012), where there was a decrease in TH in the striatum and a loss of dopamine neurons in the substantia nigra six months after mPFF injection in mice. There are a couple of possible explanations for this discrepancy since

we do observe that the PFFs are acting as shown previously in the literature. One is that the mouse strain used is more resistant to TH loss by increased α-syn load, possibly due to lower levels of endogenous α-syn or then some other unknown mechanism. In the abovementioned paper the authors used the strain C57BL6/C3H F1 (Luk *et al.*, 2012), which is a mix between C57BL/6J females and C3H/HeJ males. According to The Jackson Laboratory where the mice were obtained, this strain may "create or enhance expression of polygenic diseases", however there is currently no further information about α-syn expression in this strain (The Jackson Laboratory, 2018). Another explanation is that a longer time point was needed to observe changes. Further studies would be needed to confirm either of these explanations.

Since in the model we do have some motor behaviour changes, we tested CDNF as a possible therapy. In the instances where we do see a deficit, in this case in cylinder test in mice and rats and the coat hanger test in mice, CDNF was able to restore the behaviour. We have some hints for how this may be occurring from our *in vitro* data (publication III). First, we observed an interaction between α-syn and CDNF *in vitro* using a microscale thermophoresis assay, indicating that CDNF may be acting directly on α-syn to exert effects. Data from primary mouse cortical neurons showed that CDNF decreased the level of pS129 α-syn and in primary mouse hippocampal culture CDNF reduced the internalization of the fibrils. However, in neurons treated with PFFs, CDNF increased the number of cells that were positive for pS129; there was no effect observed *in vivo*. Although in these neuronal culture experiments there was no cell loss, we cannot rule out a functional deficit. However, our conflicting results with the fibrils may be indicative that this is not a physiological situation since in the cell-based experiments we administered the PFFs via viral overexpression or transient transfection and this likely does not represent expected levels. Although in this case, we observe effects with CDNF and the PFF model which could be related to a direct interaction with α-syn. As mentioned, there have been fewer studies with CDNF, but we can use MANF as a proxy for what may be occurring with CDNF since they share 59% amino acid identity (Lindholm *et al.*, 2007). Both proteins have an ER retention signal at the C-terminus, and with MANF it has been shown that expression increases with activators of the UPR (Apostolou *et al.*, 2008). Aggregation of α-syn has been shown to correlate with activation of the UPR in relation to the ER in human and animal models of Parkinson's disease (Hoozemans *et al.*, 2007; Bellucci *et al.*, 2011b), and it may be that CDNF is acting in this manner, either directly or indirectly (Arancibia *et al.*, 2018). For MANF, there are published studies indicating that this could be occurring. MANF is able to protect cells from ER stress in a calcium-dependent manner, possibly via direct interaction with the ER chaperone GRP78 (glucose-regulated protein 78) (Glembotski *et al.*, 2012), and related to this, GRP78 has been shown to bind to the mutant prion protein at the ER, and consequently mediate its degradation at the proteasome (Jin *et al.*, 2000). If we consider that α-syn may be acting in a similar way to the prion protein, as has been described previously (Brundin & Melki, 2017), it is possible that MANF, acting as a cofactor to GRP78, would have a similar effect in our model. Additionally, MANF has been shown to be upregulated in a model utilizing mutated matrilin-3, a protein that is aggregated in models of multiple epiphyseal dysplasia (Hartley *et al.*, 2013).

Though the disease is not related to Parkinson's disease, mutated matrilin-3 causes ER stress and activation of the UPR (Nundlall *et al.*, 2010). In the aforementioned study, MANF was shown to form a complex with matrilin-3 that included GRP78. Based on GRP78 and/or MANF's involvement with prion and aggregating proteins, it is possible that CDNF may act in the same way with α-syn. Further studies are certainly needed to ascertain which effects CDNF may be having in relation to α-syn.

6.3 EFFECTS OF CDNF INJECTED TO THE SUBSTANTIA NIGRA IN 6-OHDA AND NAÏVE RATS (IV)

We used the toxin 6-OHDA to lesion the dopamine neurons of the striatum of rats and tested CDNF injections to the substantia nigra alone or in combination to the substantia nigra and the striatum. While we observed some functional recovery on behaviour with CDNF, it was unable to restore the TH+ fibres, TH+ neurons, or DAT fibres in the brain (unpublished data). At 12 weeks, when the immunohistochemistry measurements were performed, only the combination of striatal and nigral CDNF restored the behaviour on amphetamine-induced rotations, not the nigral injection alone. Likely, CDNF given to substantia nigra was not a robust enough treatment to restore behaviour at this time, especially since striatal administration of CDNF has been successful in the rat 6-OHDA model (Lindholm *et al.*, 2007; Voutilainen *et al.*, 2011). Since at 12 weeks there was no restoration of TH or DAT with either CDNF treatment, we can conclude that it was not working here. To compare to the first study (Lindholm *et al.*, 2007), possible reasons for the discrepancy are that a single injection of 6-OHDA was used, rather than administered between two sites, and CDNF was administered four weeks after the lesion instead of two, as we have used here. For the second study mentioned above where CDNF was successful in the striatum (Voutilainen *et al.*, 2011), a chronic infusion paradigm was used where CDNF was continuously administered to the striatum of rats over a two-week period, not as a single injection as we have done in this case. Additionally, the 6-OHDA injection paradigm we used here can cause increased variation, and we have since switched to a more stable lesion paradigm where 3μg or 6μg is distributed between 3 sites, since the 20μg of 6-OHDA distributed equally to two sites has been shown to be less stable (Penttinen *et al.*, 2016). Examining the data more closely indicates that some of the control rats may not have a severe lesion, in other words their striatal TH optical density is between approximately 50% and 75%, and therefore we cannot rule out that the effects observed for CDNF here are due to a smaller lesion, rather than a therapeutic effect. It is possible that for these reasons there was no significant restoration of dopamine neurons in this study with CDNF.

We also characterized nigral injection of CDNF to naïve rats (publication IV). Striatal administration of CDNF was thoroughly characterized previously (Matlik *et al.*, 2017), where it was transported retrogradely to the substantia nigra after injection. Inside the striatum it was shown to be present both extracellularly and in the cytoplasm, and interestingly, it was detected inside substantia nigra TH+ neurons of naïve rats. Radiolabelled CDNF has also been shown to be transported retrogradely

after striatal injection to non-lesioned rats within 24 hours (Voutilainen *et al.*, 2011). We were able to repeat the latter result where radiolabelled CDNF spread to the cortex, both sides of the striatum, and the hippocampus after injection to the striatum after 24 hours. For our study, CDNF was injected to the substantia nigra and it remained in the ipsilateral side and only diffused as far as the STN. CDNF did not seem to be transported or diffused to the striatum here, which is in line with the immunohistochemistry data. When unlabelled protein was used to block the transport of radiolabelled CDNF after nigral injection, neither the 2000- nor the 10000-molar excess of unlabelled protein could block the diffusion of CDNF to the STN. Therefore, we can conclude that CDNF is not undergoing active anterograde transport to the striatum, but rather passive diffusion after nigral administration. Interestingly, only radiolabelled CDNF was present in the brain regions 24 hours after the injection. The discrepancy between the radioactivity measurements and the immunohistochemistry results could be due to the limit of detection for the antibody used. Although there was no anterograde transport of CDNF from substantia nigra to striatum, we did observe similar diffusion of CDNF around the midbrain. This occurred at 2 and 6 hours, but not 24 hours, possibly due to CDNF's half-life of 5.5 hours (Matlik *et al.*, 2017), and that it could have already diffused significantly and therefore was not detectable by immunohistochemistry at the lower concentrations present at 24 hours. Also, since CDNF diffused farther when it was diluted to a higher volume, this could increase the areas where CDNF is able to exert its effects, as has been observed with GDNF (Taylor *et al.*, 2013). Here, we also measured qualitatively whether CDNF was being taken up to TH+ or PV+ neurons of the substantia nigra after injection, since it has been shown with TH in the striatum as mentioned above. Using confocal microscopy, we observed CDNF inside TH+ neurons as well as around them in the substantia nigra area six hours after injection. However, we found that fewer PV+ neurons had CDNF puncta, and it was diffused around the cells. PV was chosen as another neuronal type since they are important in Parkinson's (Bernacer *et al.*, 2012). From these results, it is possible to conclude that CDNF may be acting intracellularly in TH+ neurons and extracellularly at both TH+ and PV+ neurons, though we cannot conclude that it is acting at all here, since we have not elucidated a cellular mechanism of how CDNF may affect dopamine neuron survival or function. In general, though CDNF did not have a positive effect in the 6-OHDA model in this particular case, we were able to characterize the effects of CDNF at the substantia nigra in order to complement ongoing preclinical and clinical studies.

6.4 COMPARISON OF PARKINSON'S DISEASE ANIMAL MODELS

Clearly, there is a large variety of Parkinson's animal models to choose from: toxin, genetic, viral overexpression, preformed fibrils, or a combination. One is not necessarily better than the other, nor is there a perfect animal model to recapitulate the human disease. In this thesis, we used four models: 6-OHDA, lactacystin, AAV, and PFF. Each of these have advantages and disadvantages, and a researcher may choose one based on

their own specific needs. Table 6 lists the main advantages and disadvantages of some popular Parkinson's disease models.

Table 6. *This table summarizes the main advantages and disadvantages to popularly used animal models in how they best recapitulate sporadic Parkinson's disease in humans.*

Model	Advantage	Disadvantage
6-OHDA or MPTP	Fast onset, selectively destroys dopamine neurons	No α-synuclein present
Lactacystin	α-synuclein present, dopamine neuron degeneration, and behavioural deficits	Protein buildup is not α-synuclein-specific
Genetic	Familial forms can be studied	Inconsistent dopamine degeneration, lack of α-synuclein present, highly dependent on mouse strain
Adeno-associated viral vector	α-synuclein present, dopamine neuron degeneration, and behavioural deficits	Does not recapitulate sporadic human disease, problems with unspecific protein overexpression
Preformed fibrils	Model of progressive Lewy pathology spreading, slow onset	Inconsistent outcomes in behaviour and dopamine neuron loss

There is currently no single model that would perfectly encapsulate the dopamine neuron degeneration, loss or dysfunction in other neuronal systems such as cholinergic, motor and non-motor symptoms, and the presence of progressive α-syn aggregation that is observed in sporadic human Parkinson's disease. Though several come close, and we cannot discount the work that has already been done in order to find a suitable model to test therapies on and discover the cause of the disease. However, continuous work is likely needed to further characterize and test new and existing models for Parkinson's disease, whether they are animal, cell, or some form of patient-derived samples.

7 CONCLUSIONS

The aim of this thesis was to use α-syn to model Parkinson's disease in rodents to test CDNF and other potential therapies on. The main conclusions from the studies are as follows:

1. Overexpression of human wild-type α-syn via AAV to model Parkinson's disease in rats results in TH downregulation and mild behavioural deficits similar to the literature, however there is high variation between animals and typical outcome measures do not correlate. Importantly, control AAVs also result in downregulation of TH and make it difficult to conclude that the effects in this model are specific to α-syn.

2. When α-syn PFFs are injected into the striatum of rodents, there is spread of pS129 α-syn to anatomically connected areas such as cortex and substantia nigra pars compacta. There are mild behavioural effects that can be ameliorated by CDNF, however there is no loss of TH in the striatum.

3. Injection of CDNF to the substantia nigra of naïve rats results in its widespread diffusion in the midbrain, including the STN, and CDNF is taken up by TH+ cells of the substantia nigra. However, there is no evidence of anterograde transport of CDNF from the substantia nigra to the striatum.

4. Lactacystin, a proteasomal inhibitor, administered to the substantia nigra of mice results in unilateral increase of α-syn, degeneration of the nigrostriatal dopamine system, behavioural deficits, and increase in neuroinflammatory markers, thus repeating what has been published for this model.

In general, the α-syn rodent Parkinson's models were modelled here relatively successfully, with the caveat that caution needs to be taken when setting up these models and proper positive and negative controls are needed. Additionally, CDNF was tested to complement ongoing clinical trials in Parkinson's disease.

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